PENICILLIN RESISTANT STAPHYLOCOCCI

INOCULUM EFFECT

by

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INTRODUCTION

The majority of the staphylococci are able to mutate or adapt to living in concentrations of penicillin that would have originally inhibited their growth. This adaptation or mutation is only possible if the staphylococci are originally exposed to sub-inhibitory concentrations of penicillin. There is also another type of staphylococci that are resistant to the action of penicillin. These strains are usually found in vivo and are penicillinase producers. Penicillinase is an enzyme that destroys the activity of penicillin.

All staphylococci bind penicillin, some to a lesser extent than others. Staphylococci that produce penicillinase exhibit what is called the "inoculum effect." This effect is demonstrated by the fact that this type of staphylococci gives no clear cut pattern of penicillin sensitivity. The greater the inoculum, the greater the resistance of these bacteria to penicillin.

In order to better understand this "inoculum effect" this study was designed to: (1) determine frequency of organisms demonstrating this effect; (2) correlate, if possible, phage type and "inoculum effect;" and (3) investigate some of the possible mechanisms of this effect.

REVIEW OF THE LITERATURE

I. HISTORY OF PENICILLIN

Fleming, during experiments in 1928, noted that colonies of staphylococci growing near a mold were undergoing lysis. He later tested the effect of filtrates of broth cultures of this mold on various organisms. This agent which he called penicillin was found to have a very low toxicity for experimental animals, but killed several species of microorganisms. It was not until eleven years later that a chemical purification of this substance demonstrated that systemic administration was possible. Although this early penicillin was very impure, it would inhibit the growth of staphylococci when the drug was diluted several hundred thousands and showed only slight toxic effects in animals (Chain et al., 1940). When a strain of Staphylococcus aureus was used that was resistant to penicillin in vitro, the therapeutic results in animals were negative (Warner et al., 1945).

The first clinical trials of systemic penicillin were carried out early in 1941 at Oxford. It was highly successful when compared to all other forms of treatment of staphylococcal infections. Except for penicillin-resistant staphylococci (Fleming, 1942), the therapeutic results were favorable when adequate blood concentrations were reached.

II. METHODS OF PENICILLIN ASSAY

Hobby et al. (1942) showed that penicillin exerts a definite bactericidal action on staphylococci and other organisms. The Oxford strain of Staphylococcus aureus was introduced as a standard strain of bacteria for standardization of the unit of penicillin. It was also used for the estimation of the penicillin concentration in biological fluids. The penicillin concentration of blood was measured using the tube method and inhibition of the growth of staphylococci as an indicator (Fleming, 1943; Hildick-Smith et al., 1949). The zones of inhibition around cups containing penicillin, when placed on agar plates, were related to the penicillin concentration (Heatly, 1944b). Other biologic assays were based on the turbidimetric readings of growth of organisms obtained with varying concentration of the drug (Foster, 1942; Foster et al., 1943; Green, 1946). Later chemical methods for determining penicillin concentration were devised (Geronimus et al., 1957). Direct measurement of the breakdown of penicillin to penicilloic acid can be accomplished by use of a Warburg apparatus. The mono-sodium salt of penicilloic acid will liberate one mole of carbon dioxide from a solution of bicarbonate. By utilizing the same breakdown into penicilloic acid, an iodometric assay for penicillin can also be used for determining the penicillin

concentration (Mundell et al., 1946; Alicino, 1946). This assay is based on the fact that iodine will only combine with inactivated penicillin. Both of the above methods can be used to assay for penicillinase (see below) activity.

III. MODE OF ACTION OF PENICILLIN

It is difficult to determine the metabolic system involved in the action of drugs. The mode of action of penicillin is further complicated by its selective toxicity for susceptible bacterial species. The metabolic activities of a cell are inter-related and an interference of one system may interfere with many related metabolic pathways. The isolation of an enzyme system from a cell may offer a better insight to this problem. If this system is inactivated in vitro and can be proved to act in the organism in a similar manner, the action of the drug used for the inactivation process may be assumed to act in a like manner in the cell. The first observation on the nature of the antibacterial action of penicillin was made by Fleming (1928). By use of a crude preparation of penicillin and inoculating this with a small number of staphylococci, he observed that after a brief period of multiplication, the count of viable organisms declined and all of the tubes became sterile. Abraham et al. (1941), on the other hand, thought that the effect of penicillin was mainly bacteriostatic. They used a twenty-four

hour broth culture of a staphylococcus and demonstrated no effect on the oxygen up-take of the cells. Hobby et al. (1942) confirmed Fleming's original observation that penicillin exerts a definite bactericidal action on staphylococci as well as on other organisms. They found that the number of viable organisms decreased at such a rate that the logarithm of the number of surviving organisms was a linear function of time. The rate of destruction increases with an increase in penicillin concentration up to a maximum at which higher concentrations had no increased killing effect. The bactericidal action of penicillin was exerted only under conditions which allowed growth of the organisms. A resting population of microorganisms is not killed by penicillin. The action of penicillin on staphylococci has been verified by many workers (Rammelkamp et al., 1943; Lee et al., 1944; Rantz et al., 1944a; Rantz et al., 1944b; Todd, 1945).

The time required for penicillin to produce an irreversible amount of damage to bacterial cells has been investigated. When a culture in the logrithmic phase of growth is exposed to penicillin in low concentrations and then washed free of the drug, the amount of damage depends on the sensitivity of the strain used, the amount of time the penicillin was in contact with the organisms, and the concentration of penicillin used. When a high concentration of penicillin is used, only a very short time may lapse before

killing of the organisms results. With still higher concentration of the antibiotic, there is immediate destruction of the organisms (Marsh et al., 1946; Parker et al., 1946). As might be expected, the damage to the cells at low temperatures is reduced for a given concentration of penicillin (Parker, 1953).

It is difficult to completely sterilize a nutrient broth culture of staphylococci by the use of penicillin (Hobby et al., 1942; Rammelkamp et al., 1943). Even after lysis, a small number of staphylococci in the culture fluid remain viable (Rantz et al., 1944a). The individual cells escape the action of the penicillin, have a high natural resistance to the penicillin, acquire it through contact when exposed to penicillin, or are in a growth phase in which they are insusceptible to its action. The last view seems more tenable because the remaining progeny showed no increased resistance to penicillin.

Lederberg (1957) is of the opinion that the essential mode of action of penicillin is in the inhibition of cell wall synthesis, thus producing an osmotic fragility in the sensitive bacteria. He suggests that the biochemical target of penicillin is a specific wall-building polymerase, with the differences in the antibiotic activity being due to the different make-up of the bacterial cell wall among different species of bacteria.

Early attempts to demonstrate that penicillin is actually used up in its action on bacteria failed because the biological assay methods were not sensitive enough to detect the minute amount of penicillin involved in the reaction (Hobby et al., 1942). Rowley et al. (1948) used a preparation of radioactive penicillin and demonstrated that all bacteria fix penicillin, however, more penicillin was fixed by sensitive bacteria than by resistant ones, and the uptake of the labeled penicillin was increased proportionally to the growth of the bacteria. Cooper et al. (1949) found that the penicillin was firmly bound and could not be removed by washing or incubating with acids or alkali. Maass et al. (1949a; 1949b) demonstrated that specifically absorbed and bound penicillin which could not be dislodged either by washing or changing the equilibrium using non-radioactive penicillin, was 750 molecules per cell. It has been suggested that the growth that occurs when penicillin treated cells are transferred to penicillin free medium is due to the resynthesis of an exhausted metabolite, and it is possible that this resynthesis of the penicillin binding component is the chief requisite for renewed growth. It was found experimentally that the cells in which all the penicillin binding component had been blocked resynthesize the new component two or three times as rapidly as the cell substance is synthesized. The bound penicillin is not dislodged with renewed growth, but

remains in the daughter cells in correspondingly reduced concentrations. According to the views of Maass et al. (1949b), the lowest bacteriostatic penicillin concentrations are those in which blocking of the penicillin binding component is slightly more rapid than its resynthesis by the cell.

Cooper et al. (1949) attempted to locate the penicillin binding component after disrupting. He treated staphylococci by shaking them with glass beads. By treating the staphylococci with formalin before shaking the greater part of the radioactivity was found in the cell wall. Ordinarily cell wall preparations were found to bind penicillin several times more readily, on the dry weight basis, than intact staphylococci. No penicillin binding component could be detected by using an electro-dialysis method for removing unbound penicillin (Few et al., 1953). Daniel et al. (1954) found that in cell extracts, the binding component was heat labile, but practically unaffected by treatment with trypsin and chymotrypsin.

The correlation between penicillin binding component and the resistance of various strains of staphylococci is somewhat complex. When resistant variants are compared with their sensitive parent strains they are found to bind more, less, or equal amounts of penicillin (Eagle, 1954a; 1954b). These variants escape the action of penicillin through an action not known (Eagle, 1954c). Generally, however, there

is a correlation between the combining afinity and the resistance to the drug. The resistant strains tend to bind less drug than the sensitive ones. The penicillin binding capacity of cell free extracts also correlates with the sensitivity of the strain. Although great differences may be required in the environmental concentration of penicillin to inhibit the growth of different strains, there are only small differences in the number of molecules bound per cell (Eagle et al., 1955).

On the whole, the evidence is in favor of the hypothesis that the actual determination of penicillin sensitivity is the combining affinity of a vulnerable cell component which is functionally inactivated by the combination. The metabolic pathways of these combinations are not yet clear. Little is known about the actual nature of the union between the penicillin binding component and the antibiotic.

The available evidence suggests that penicillin interferes with some metabolic system which is most active during growth. This interference appears to act at the biochemical level of the control of protein synthesis. High concentrations of penicillin have been used to bring about complete inhibition of all protein synthesis, but these high concentrations of penicillin are not needed to cause bacteriostasis. The inhibition of one specific important protein would do this.

IV. PENICILLIN RESISTANCE

Abraham et al. (1941) showed that the resistance of the Oxford strain of staphylococcus used for penicillin assay could be increased a thousandfold by growing it serially in sub-inhibitory concentration of the drug. Numerous other workers have confirmed this finding using other strains of Staphylococcus aureus (Rammelkamp et al., 1942; Rake et al., 1944; Spink et al., 1944b; Bondi et al., 1944; Bondi et al., 1945; Chain et al., 1945; Levine et al., 1945; Spink et al., 1947).

Demerec (1945; 1948) showed that resistance to penicillin is not induced by the drug but originates through genetic changes comparable to gene mutations. He demonstrated that a sensitive population of staphylococci is composed of individuals with varying penicillin sensitivity. Lederberg et al., (1952) showed that when a sensitive staphylococcal population is grown in the absence of the drug and then a replica made on nutrient agar containing penicillin, the colonies arising from resistant mutants can be demonstrated. This theory of drug resistance has received no serious challenge; it not only fits all known facts but aids in explaining how adaption might arise.

Strains have been described that resist several thousand units of penicillin (Gale, 1947; Bellamy et al.,

1948a; Bellamy et al., 1948b; Klimek, et al., 1948). Further support of the mutation theory is the fact that back mutations to sensitivity have been observed. Barber (1953a) has shown that different types of drug resistant mutants can be obtained. She has described penicillin dependent variants (Barber, 1953b). These penicillin dependent variants are capable of growing only in the presence of penicillin. Hamburger et al., (1960) reported the discovery of a penicillin sensitive Staphylococcus aureus after experimental endocarditis had been produced with a penicillin resistant strain. Both staphylococci had identical phage types suggesting a reverse mutation.

It has been argued that penicillin resistant strains obtained in vitro are different from those obtained in vivo because the in vivo resistant strains produce penicillinase. In argument against this, Szybalski (1953a) demonstrated that occasional in vitro mutants also produce penicillinase.

From the practical point of view the mutational origin of resistant strains has an important bearing on the clinical application of antibiotics. The usefulness of an antibiotic is closely related to the frequency of resistant pathogens and their incidence in infection. It is therefore important to attempt to avoid the development of resistant strains during therapy. The work of Demerec has shown that as far as penicillin and staphylococci are concerned this is a

relatively simple matter. Since the resistance develops in steps, and most of the sensitive strains are quite sensitive to penicillin, all that is required to avoid the development of resistant strains is to maintain the dose of penicillin high enough to eliminate the development of the first step mutants. It is also important to maintain the effective concentration as long as the infection persists.

Although early attempts to produce penicillin resistant staphylococci in experimental animals treated with the drug failed (McKee et al., 1943; Rake et al., 1944; North et al., 1946), it soon became obvious that staphylococci from patients treated with penicillin may show greatly increased resistance (Rammelkamp et al., 1942; Florey et al., 1943; Taylor et al., 1944; Spink et al., 1944b; Blair et al., 1946; North et al., 1946).

Penicillin resistant staphylococci undoubtedly existed before the introduction of the drug, and it seems likely that the increased incidence of resistant strains was due to the elimination of the susceptible organisms by the widespread use of penicillin and their replacement by an originally very small minority of naturally resistant organisms (Barber, 1947b).

V. STAPHYLOCOCCAL PENICILLINASE

Abraham et al. (1940) were the first to show that

certain bacteria produce an enzyme capable of destroying penicillin. This finding was at once associated with the idea of penicillin resistance of staphylococci, and a search for penicillinase producing organisms was made among sensitive and resistant staphylococci obtained in vitro. The results were at first negative (Abraham et al., 1941). Kirby (1944b; 1945a), however, compared sensitive and naturally resistant strains and found penicillinase in the latter group. Although penicillinase influences the sensitivity of a strain, it is not the sole factor in resistance. Gots (1945a) found a considerable amount of the enzyme was produced by resistant strains obtained from patients and this was confirmed by Bondi et al. (1945). Bellamy et al. (1948b) claimed to have found a highly resistant variant which produced extracellular penicillinase when grown in the presence of penicillin, but this strain had some remarkable properties; it was a gram-negative rod with biological properties very different from staphylococci. Kirby (1945b) first observed in vitro variants producing penicillinase. Segalove (1947) worked with a strain which had a high initial resistance but produced no detectable penicillinase; but on prolonged cultivation in the presence of penicillin, its resistance was considerably increased and penicillinase production was demonstrated.

Penicillin resistant strains obtained from patients

are always capable of producing penicillinase (Spink et al., 1945b), while the in vitro penicillin resistant variants seldom produce the enzyme. Bondi et al. (1948a) showed that some individual cells of a penicillinase producing strain may be highly sensitive to the drug. By using the gradient plate, Szybalski (1953) provided direct proof of the above and of the mutational origin of penicillinase producing staphylococci.

VI. PENICILLINASE ASSAYS

The testing of organisms for penicillinase production is based on the observation that the activity of penicillin has been reduced or destroyed. Whole cultures, filtrates, or liberated intracellular penicillinase can be incubated with penicillin. Gots (1945b) incorporated in a solid medium a small amount of penicillin and a large inoculum of penicillin sensitive staphylococci. If the strain produced penicillinase, the drug was destroyed in its vicinity, allowing the sensitive staphylococci to grow around the penicillinase producing strain in the form of satellite colonies. El Ghoroury (1952) reported another penicillinase assay method which employed uniform inoculation of a plate with a sensitive organism. Paper discs impregnated with penicillin were then placed on the surface. One served as a control, and to the others a loopful of the broth culture of

the strains to be tested was added. The diminution of the zone of inhibition indicated penicillinase production. The previous methods for penicillin assay (manometric and iodometric) may also be used for penicillinase assays.

VII. METHODS OF PENICILLINASE PREPARATION

Kirby (1944b; 1945b) used the method previously introduced for the purification of penicillinase produced by the paracolon bacilli. He prepared penicillinase powder from staphylococci by precipitation with acetone, treating the precipitate with ether and drying it in a vacuum. Spink et al. (1947) used the same method. These preparations are referred to as intracellular penicillinase, but the powder is composed of defatted, but otherwise intact, bacterial cells. It is not a pure penicillinase enzyme, but rather the organisms are treated to reveal presence in the cell. Gilson et al. (1948) used a modification of this method which gives higher yields, by the use of acetone extraction in the cold at -20°C followed by similar treatment with ether. They obtained a highly active preparation but were unable to obtain an extract of the enzyme in water at pH 7.0. Since penicillinase readily diffuses into the cultural medium, an extracellular penicillinase was suggested (Czekalowski, 1950). Housewright et al. (1947) stated that their strain of Staphylococcus aureus produced extracellular penicillinase

in concentration comparable to that of Bacillus cereus. Lippman et al. (1957) have shown that staphylococcal penicillinase is an inducible enzyme similar to that found in many species of the genus Bacillus.

Gilson et al. (1948) showed that the rate of destruction of penicillin by staphylococcal penicillinase is a function of time, temperature, and concentration of the enzyme. They defined one unit of penicillinase as the quantity required to reduce the concentration of solution of penicillin from 10 units per ml. to 5 units per ml. in thirty minutes at 37°C, at a pH 7.0.

VIII. PROPERTIES OF PENICILLINASE

Penicillinase is a protein which is non-dialysable and destroyed by heat and certain proteolytic enzymes. In solution, it is completely destroyed in five minutes at 56°C (Kirby, 1945b). Staphylococcal penicillinase is stable at neutral pH and completely destroyed by incubation, when incubated at 37°C for four hours at pH 9.0. It loses half its activity when incubated at 37°C for thirty minutes at pH 2.0 or pH 11.0, or four hours at pH 3.0 (Spink et al., 1947). Sodium azide, a metabolic inhibitor, has no direct effect on staphylococcal penicillinase but suppresses the production of the enzyme by the cocci (Bondi et al., 1948b).

Penicillinase, like other enzymes, is antigenic.

Housewright et al. (1947) produced an antiserum in rabbits to Bacillus cereus penicillinase which neutralized its action. They have shown that this serum also inhibited the extra-cellular penicillinase of their atypical strain of Staphylococcus aureus and concluded that the two enzymes were immunologically similar. Other workers have not been able to confirm this immunologic similarity between the two penicillinases. Perlstein et al. (1945a) reported that the injection of penicillinase intravenously into rabbits produced an antiserum which produced a precipitin reaction with the enzyme at a 1:256 dilution. In a later paper, these authors (Perlstein et al., 1945b) showed that penicillinase antiserum protected penicillin from the destruction of penicillinase.

Wick et al. (1959) reported that anti-penicillinase serum was beneficial in preventing infection by penicillinase producing staphylococci if the antibiotic and antibody are present either prior to, or shortly after infection.

The action of penicillinase is hydrolytic in nature. It, like dilute alkali, hydrolyzes penicillin to penicilloic acid. It can, therefore, be considered to be a peptidase which opens the beta lactam ring (Florey et al., 1949).

Penicillinase is used in diagnostic work for tests of sterility of penicillin preparations, and in the isolation of organisms from the blood, tissues, pus, and secretions from

patients being treated with penicillin. The enzyme is added to the culture media to destroy the penicillin which would otherwise prevent the growth of viable organisms (Kirby, 1944a).

IX. PROPERTIES OF RESISTANT STRAINS OF STAPHYLOCOCCI

By comparing resistant strains with their sensitive parent strains, many workers have reported that the resistant strains show a reduced rate of growth and fermentation (Abraham et al., 1941; Rake et al., 1944; Blair et al., 1946; North et al., 1946; Bellamy et al., 1948b).

Stone (1951) attempted to show a diffusion of essential growth factors from an induced penicillin resistant variant to the sensitive parent strain. Thiele et al. (1959) reported that anaerobic conditions did not change the penicillin sensitivity or penicillinase production of a resistant strain of Staphylococcus aureus. While a sensitive strain showed a tendency to increase in penicillin resistance when grown anaerobically. Very little information is available about penicillinase producing strains that are found in natural conditions. Bondi et al. (1954) compared penicillinase producing staphylococci with penicillin sensitive strains by growing them in a medium containing salt, glucose, thiamine, nicotinic acid, and nineteen amino acids. The test

consisted of omitting each of the chemical groups singly. No consistent difference could be found between the two groups, but there was a variation between strains.

The most important characteristic of penicillin resistant staphylococci is their virulence. There is general agreement that the majority of penicillin resistant variants obtained in vitro show decreased virulence for mice (McKee et al., 1943; Rake et al., 1954; Blair et al., 1946; North et al., 1946). Evidence that these strains are more susceptible to the bactericidal action of whole blood and possibly to other defense mechanisms of the host than are ordinary staphylococci has been reported (Spink et al., 1944a; Spink et al., 1945; Spink et al., 1940). Naturally resistant penicillinase producing strains, however, possess undiminished pathogenicity to man (Lyons, 1943; North et al., 1946) and no difference in virulence could be demonstrated between such strains and virulent, sensitive staphylococci in experimental animals.

X. BACTERIOPHAGE TYPING OF STAPHYLOCOCCI

Although epidemics of staphylococcal infections comparable to the common contagious diseases do not occur, minor outbreaks in closed or semi-closed communities are sufficiently common to suggest that single epidemic strains are involved rather than random cross infection with more

than one strain. Some method of fine classification is required to recognize these strains among the vast number and varieties of staphylococci in the environment. Coagulase and serological testing give limited information but fail to provide discrimination fine enough to recognize strains isolated from widely separated places or at different times as being identical.

Burnet and Lush (1935), using four phages, demonstrated differences between strains of staphylococci. These phages were later used to investigate staphylococci obtained from acute osteomyelitis. The systematic application of phages for epidemiological investigation of staphylococcal infection was first employed by Fisk (1942a; 1942b) at a time when phage typing of typhoid bacilli was already of proven value. Fisk was able to show that the susceptibility of staphylococci was not altered by the environment: staphylococci maintained in stock culture or after repeated mouse passage still reacted to the phages in the same manner. His technique consisted of spotting phage suspensions on plate cultures of staphylococci, under test, in a given pattern. The patterns of lysis obtained were compared. In this way it was possible to show the identity of two strains even though they differed in pigmentation and hemolysin production (Fisk et al., 1944).

Wilson and Atkinson (1945) extended this work and laid the foundations of the phage typing system which with

minor modifications and additions is now used throughout the world. Some of the practical and theoretical problems involved in phage typing were discussed by Williams et al. (1952; 1953). They pointed out that this system of phage typing of staphylococci depends on the recognition of receptors that are or may be shared by different types, and strains from various sources are distinguished by their patterns of sensitivity to numerous phages.

Blair and Carr (1958) report that phage typing Staphylococcus aureus directly involved in hospital infections, 70 to 80 per cent of the reported cases fall into the phage type designated as 80/81. The other virulent phage types are found in localized outbreaks in individual hospitals. In these cases, very frequently a Staphylococcus aureus with an identical phage type as these isolated from the infections may be found in the noses and throats of the hospital personnel in the infected area. In a previous article, Blair and Carr (1953) outline the methods of phage typing they use that produces good results.

XI. BACTERIOPHAGE PRODUCTION

The production of high titer phage stocks is one of the problems faced by a person doing phage typing. Swanstrom et al. (1951) report an agar layer method for the production of high titer phage stocks. Another method by Liu (1958)

employs a cellophane plate technic. This method requires a shorter time and is reported to yield phage stocks of high titer.

White et al. (1959) described a method of phage propagation in liquid medium. They reported that by using calcium in concentrations of 0.004 M, the phage titers are similar to those obtained using the more involved agar layer methods.

According to Fussilo (1956), Staphylococcus aureus with multiple resistance to two or more antibiotics, kept their specific phage sensitivity.

XII. INOCULUM EFFECT

Kirby (1945a) noted that the sensitivity of a penicillinase producing strain of staphylococcus was dependent on the number of microorganisms in the inoculum. While a small inoculum was completely sterilized by a certain concentration of penicillin, a large inoculum grew in this concentration of penicillin. Luria (1946) showed that this observation can be brought about by two mechanisms: (1) the presence in a large inoculum of resistant bacterial mutants, and (2) the destruction of penicillin by penicillinase produced by a strain whose individual cells are sensitive to penicillin. Gilson et al. (1948), in a study of a large number of strains of staphylococci, found that the average

resistance of strains producing penicillinase was 250 times that of the others when a large inoculum was used, but only 8 times with a small inoculum. Similar observations were also made by other workers (Parker, 1946; Spink et al., 1947; and Barber, 1947a). Louria et al. (1960) report a similar inoculum effect in treatment of an experimental staphylococcal infection in mice.

MATERIALS AND METHODS

I. COLLECTION OF STAPHYLOCOCCAL STRAINS TO BE INVESTIGATED

In order to obtain a pure strain of Staphylococcus aureus, all staphylococci collected, except those where specific reference is made to a different source, were obtained from blood cultures taken from patients suffering from staphylococcal bacteremia. This source of culture helps rule out any infections due to a multiplicity of strains of staphylococci.

The following is a list of the strains which were collected. More than one culture was obtained from some of these patients; these are listed as cultures a, b, c, etc.

TABLE I

STAPHYLOCOCCAL STRAINS COLLECTED FOR INVESTIGATION

Strain	Culture	Source	Hospital	Miscellaneous
1	a	Blood	VAH (Ft. Douglas)	
1	b	Blood	VAH (Ft. Douglas)	
1	c	Blood	VAH (Ft. Douglas)	
1	d	Blood	VAH (Ft. Douglas)	
1	e	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	f	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy

TABLE I (continued)

Strain	Culture	Source	Hospital	Miscellaneous
1	g	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	h	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	i	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	j	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	k	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	l	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	m	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	n	Blood	VAH (Ft. Douglas)	Patient on Penicillin therapy
1	o	Blood	VAH (Ft. Douglas)	Autopsy culture
2	a	Blood	LDSH	
3	a	Blood	VAH (12th Avenue)	
4	a	Blood	VAH (12th Avenue)	
5	a	Blood	VAH (12th Avenue)	
6	a	Blood	LDSH	
7	a	Blood	LDSH	
8	a	Blood	LDSH	
9	a	Blood	LDSH	
9	b	Blood	LDSH	
9	c	Blood	LDSH	Kanamycin sulfate & Penicillin therapy
9	d	Blood	LDSH	Kanamycin sulfate & Penicillin therapy

TABLE I (continued)

Strain	Culture	Source	Hospital	Miscellaneous
9	e	Blood	LDSH	Kanamycin sulfate & Penicillin therapy
10	a	Blood	VAH (12th Avenue)	
11	a	Blood	LDSH	
11	b	Blood	LDSH	
11	c	Blood	LDSH	Following Penicillin therapy
11	d	Blood	LDSH	Following Penicillin therapy
11	e	Blood	LDSH	Following Penicillin therapy
12	a	Blood	LDSH	
12	b	Blood	LDSH	
12	c	Blood	LDSH	
13	a	Blood	VAH (Ft. Douglas)	
14	a	Blood	VAH (12th Avenue)	
14	b	Blood	VAH (12th Avenue)	
14	c	Blood	VAH (12th Avenue)	
15	a	Blood	VAH (12th Avenue)	Autopsy Culture
16	a	Blood	LDSH	
17	a	Blood	VAH (12th Avenue)	
18	a	Blood	LDSH	
18	b	Blood	LDSH	
19	a	Blood	VAH (12th Avenue)	

TABLE I (continued)

Strain	Culture	Source	Hospital	Miscellaneous
20	a	Blood	VAH (12th Avenue)	
20	b	Blood	VAH (12th Avenue)	Following Penicillin therapy
20	c	Blood	VAH	Following Penicillin therapy
20	d	Blood	VAH (12th Avenue)	Following Penicillin therapy
20	e	Blood	VAH (12th Avenue)	Following Penicillin therapy
21	a	Spinal Fluid	VAH (Ft. Douglas)	

To carry out penicillin assays by the cylinder cup method, Staphylococcus aureus strain 22 (ATCC #6538p) was obtained from the American Type Culture Collection.

II. MAINTENANCE OF THE STOCK STAPHYLOCOCCI CULTURES

The previously mentioned staphylococcal strains were initially isolated from the blood cultures by the following method: The blood culture broth was streaked on blood agar and three subsequent transfers made from the isolated colonies. Organisms from the final blood agar plate were transfered to Trypticase soy agar[1] slants. After incubation at 37°C for 24

[1]Baltimore Biological Laboratory, Inc., Baltimore 18, Maryland.

hours, the cultures were kept at 4°C. Subcultures were made to fresh trypticase soy agar slants every four weeks.

III. BACTERIOPHAGE TYPING OF STAPHYLOCOCCI

The international subcommittee on staphylococcal phage typing recommend (personal communication with Dr. Lewis Griffith) that the following bacteriophages be used to type staphylococci:

TABLE II

BACTERIOPHAGE USED FOR TYPING STAPHYLOCOCCI

Group 1	Group 2	Group 3	Group 4	Miscellaneous
79	3a	83	42d	71
52a	3b	73		81
52	3c	75		187
29	55	77		
		53		
		54		
		47		
		42e		
		42b		
		70		
		6		
		7		

Although the bacteriophages were classified into five groups, it was not possible to differentiate the staphylococci on this basis alone since there were many cross reactions which complicate typing.

The staphylococcal phages were obtained from Dr. Lewis Griffith, Veteran's Administration Hospital, Batavia, New York. The titer of the phage stock had been previously determined by Dr. Griffith, thus dilutions of the stock phage were made to obtain working suspensions.

The phage typing was carried out in the following manner: The staphylococcal strain to be typed was grown for six hours in trypticase soy broth at 37°C. This culture was then streaked over the surface of a previously dried trypticase soy agar plate. To the surface of these inoculated plates the different phage suspensions were added. The location on the plates of the various phage suspensions was recorded and used in the intrepretation of the results. The plates were then incubated over night at 37°C. Any plate showing 50 plaques to confluent lysis was considered a positive test. Bacterial growth with less than 50 plaques were considered to be negative or non-typeable.

IV. POUR PLATE SENSITIVITY TESTS

A 12 to 18 hour broth culture of the organism under investigation was grown at 37°C. in nutrient broth.[2] Pour plates were made using blood agar base.[2] To this medium penicillin was added in the following concentrations:

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[2]Difco Laboratories, Inc., Detroit 1, Michigan.

0.0, 0.1, 1.0, 10.0, 100.0, and 1000.0 units per ml. One ml of the following dilutions of a 12 to 18 hour broth culture of the organism under investigation was added to separate petri dishes: 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-6}; thus giving five petri dishes of varying dilutions of organisms for each concentration of penicillin. The agar medium, containing the previously mentioned concentrations of penicillin, was then poured into the petri dishes containing the organisms and the contents were thoroughly mixed. After 24 hours incubation at 37°C the plates were examined and a count of the colonies made.

A refinement was made of the pour plate method of testing for the sensitivity of staphylococcal strains to penicillin. This consisted of adjusting the concentration of penicillin to 1, 5, 10, 25, 50, 75, 100, 500, and 1000 units per ml. The concentration of organisms in the inoculum was changed to one ml of a two-fold dilution of a broth culture instead of the serial ten-fold dilutions used in the previous pour plate tests. To determine the number of organisms used in the inoculum, pour plates were made by diluting the original culture to 10^{-7} and 10^{-8}. From these counts it was possible to determine the number of organisms in the original culture. The remainder of the experiment was carried out in the same manner as the original pour plate method.

V. TESTING FOR PENICILLIN SENSITIVITY BY THE DISK METHOD

The strain under question was inoculated into nutrient broth. This broth was then incubated at 37°C for three hours. At the end of this time a cotton swab was moistened with the broth and streaked over the surface of a Mueller Hinton blood agar plate. In the center of the inoculated area, a 10 unit penicillin sensitivity disk was placed. After incubating the nutrient broth cultures for eight hours, the procedure was repeated. The Mueller Hinton blood agar plates were then placed in the incubator at 37°C for 18 hours. At the end of this time the diameter of the zones of inhibition was measured and recorded in centimeters.

VI. CHEMICAL METHOD FOR THE ASSAY OF PENICILLIN

The following method was adapted from Alicino et al. and is used by the Food and Drug Aministration: A weighed sample of Penicillin G (approximately 30 mg.) was diluted with phosphate buffer, pH6, to a concentration of approximately 1.2 mg. per ml (2,000 units per ml). Aliquots of 2.0 ml were added to each of two 125 ml glass stoppered Erlemeyer or iodine flasks. To one of these flasks was added 2.0 ml of 1 N sodium hydroxide. This flask containing the sodium hydroxide was allowed to stand at room temperature for 15 minutes. At the end of this time, 2.0 ml of 1.2 N sodium

thiosulfate previously standardized accurately against potassium iodate was added. Toward the end of the titration, one drop of starch solution or about 5.0 ml of carbon tetrachloride was added. The end point was reached when the blue color of the starch iodine complex became clear and colorless or when the carbon tetrachloride layer became colorless. To the second flask, 10 ml of the 0.01 N iodine solution was added and titrated immediately with 0.01 N sodium thiosulfate for the blank determination. The difference in titrations was divided by a factor, F, which is the number of ml of 0.01 N iodine absorbed by the 1.0 mg of sodium penicillin G working standard to obtain the concentration of unknown penicillin. The factor, F, was determined by actual standardization against the sodium penicillin G working standard, using the above method.

VII. DITCH PLATE METHOD OF TESTING FOR PENICILLIN SENSITIVITY

The plates for the Ditch Plate method were prepared by cutting a wedge of agar from the center of a Mueller Hinton blood agar plate. Agar containing penicillin was then poured into this space. A strain of alpha hemolytic streptococci was then streaked perpendicular to the ditch. A strain of Staphylococcus aureus was then streaked on either side of the Streptococci. The plates were then incubated for 18 hours

at 37°C and examined for the presence of zones of inhibition.

VIII. THE EFFECT OF SODIUM AZIDE ON THE PENICILLIN SENSITIVITY OF STAPHYLOCOCCI

Two concentrations of sodium azide were added to the nutrient broth concentration before incubation. The resulting organisms were then tested for penicillin sensitivity by the previously mentioned pour plate method. An alternate method was the incorporation of the azide into the agar of the pour plate penicillin sensitivity test. The third method of investigation was a combination of the sodium azide in both the agar and broth. The results were determined by colony counts as in the pour plate method.

IX. OPTICAL DENSITY CURVE OF STAPHYLOCOCCAL STRAIN #11-d

The Optical Density curve of strain #11-d was accomplished by inoculating nutrient broth with the organism under test. The resultant bacterial cells when incubated at 37°C was determined by plate counts and the transmission of light measured at 6400 Å in a Leitz photoelectric colorimeter.

X. DETERMINATION OF THE EFFECT OF PENICILLIN ON LAG PERIODS

To determine the effect of penicillin on the length of

lag periods, nutrient broth cultures were incubated in the presence of penicillin at a concentration of 50 units of penicillin per ml. The number of organisms inoculated into the broth was varied and controlled by pour plates as in previous experiments. Samples were taken at various times and pour plates made to determine the number of organisms.

XI. CYLINDER PLATE TWO DOSE PROCEDURE FOR DETERMINING CONCENTRATION OF PENICILLIN USING STAPHYLOCOCCUS AUREUS (ATCC 6538p)

This cylinder plate assay was run using the method of the Food and Drug Administration. Four plates were used for each sample. One cylinder on each plate was filled with a standard concentration of 1.0 unit per ml. To another cylinder, a 0.25 unit standard solution was added. To the remaining two cylinders on each plate was added the estimated concentration of 1.0 unit per ml and 0.25 unit per ml of the sample under test. The plates were carefully placed in racks and incubated at 37°C for 16 to 18 hours. After incubation the diameter of each area of inhibition was measured and recorded.

The potency of the unknown was determined by the use of the accompanying chart (Figure 1). To use the chart (Figure 1) for estimating the potency, two values were

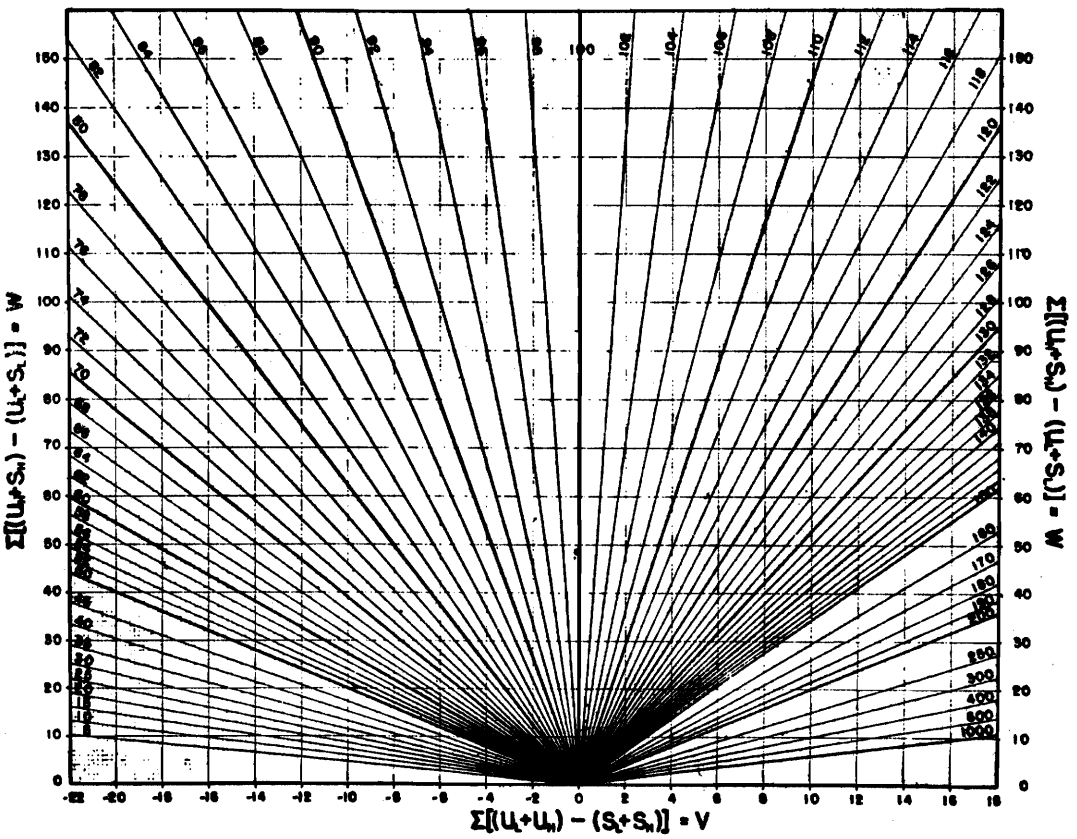

Figure 1. Penicillin assay chart

required. The values were given the letters V and W. For each plate these two values were calculated:

$$V = (ul + uh) - (sl + sh)$$

$$W = (uh + sh) - (ul + sl)$$

Where sh and sl were the diameters of the zones of inhibition in millimeters of the 1.0 unit and 0.25 unit concentration of the standard, respectively, and uh and ul referred similarly to the concentrations of the sample under test, the value V was the sum of the v values for all of the plates. W was the sum of the w values for all of the plates. To estimate the potency, the point on the chart corresponding to the values of V and W was located and read from the radial lines on the chart.

XII. AMOUNT OF PENICILLIN ADSORBED TO THE STAPHYLOCOCCAL CELLS

The adsorption experiments were performed as follows: A suspension of bacterial cells was added to a solution of penicillin at a concentration of 50 units per ml. This mixture was incubated in the refrigerator at 4°C. At 30 minutes and 60 minutes, the mixture was centrifuged at approximately 20,000 G in a Sorvall centrifuge (model SSI) for 15 minutes to free the supernatant fluid of bacterial cells. A portion of the fluid was then removed and assayed

for penicillin by the cylinder plate method. Varying concentrations of different strains of organisms were used.

The affect of Mg^{++} and Ca^{++} on the adsorption of penicillin was studied in a manner similar to the method just mentioned. The only exception being that instead of saline being used to suspend the organisms and penicillin, a solution containing Mg^{++} and Ca^{++} (0.05 and 0.005 M concentration) was substituted for the saline.

XIII. THE RATE OF DESTRUCTION OF PENICILLIN BY STAPHYLOCOCCAL STRAINS AT 37°C

By using the following methods, the rate of destruction of penicillin by staphylococci was studied: Broth containing 50 units of penicillin per ml was inoculated with varying concentrations of organisms. This mixture was then incubated at 37°C. At given time intervals, aliquots were removed and boiled for 15 minutes to inactivate the penicillinase produced, if any, by the organisms. Dilutions were made of aliquots from the inactivated material to obtain a concentration of penicillin in the range measurable by the plate cylinder method of assay. An assay of the remaining penicillin was made by this method. Controls were used to demonstrate no loss of penicillin activity by boiling for 15 minutes.

EXPERIMENTAL RESULTS

In order to obtain some insight into the problem of the inoculum effect, it was thought that the strains of Staphylococcus aureus illustrating this effect should be grouped if possible. The only feasible method for grouping staphylococci is bacteriophage typing. The results of the staphylococcal bacteriophage typing are listed below in Table III.

TABLE III

BACTERIOPHAGE TYPES OF STRAINS INVESTIGATED

Strain	Demonstration of Inoculum Effect	Phage Type
1	Positive	Untypeable
2	Negative	Untypeable
3	Negative	Untypeable
4	Positive	Untypeable
5	Positive	Untypeable
6	Positive	Untypeable
7	Positive	Untypeable
8	Negative	Untypeable
9	Positive	Untypeable
10	Positive	Untypeable
11	Positive	Untypeable
12	Positive	81
13	Positive	81
14	Positive	81
15	Positive	80/81
16	Positive	80/81
17	Positive	Untypeable
18	Positive	Untypeable
19	Positive	4
20	Positive	47/54/75
21	Positive	4/53/6/75/77
22	Negative	42D

In all of the previous strains, the different cultures of each strain had the same phage type as the initial isolate, thus partially demonstrating the purity of the strains isolated.

From the results of Table III it was not possible to draw any conclusions as to the phage grouping of staphylococci illustrating the inoculum effect.

Table IV illustrates the results when strain #1 was tested for penicillin sensitivity by using the disk method:

TABLE IV

DISK SENSITIVITY TEST OF 3 AND 8 HOUR CULTURES

Strain #1 Culture	3 hour culture Zone of inhibition Diameter in cm.	8 hour culture Zone of inhibition Diameter in cm.
a	1.5 cm.	0.7 cm.
b	1.5 cm.	0.7 cm.
c	3.75cm.	3.2 cm.
d	1.5 cm.	0.7 cm.
e	1.5 cm.	0.8 cm.
f	1.3 cm.	0.7 cm.
g	1.2 cm.	0.7 cm.
h	1.6 cm.	0.8 cm.
i	1.3 cm.	0.7 cm.
j	1.3 cm.	0.6 cm.
k	1.4 cm.	0.6 cm.
l	1.3 cm.	0.6 cm.
m	2.0 cm.	0.6 cm.
n	3.0 cm.	0.7 cm.
o	3.5 cm.	0.9 cm.

From this experiment, it was noted that the age of the broth culture had an effect on the penicillin sensitivity of the organism under investigation. This was probably due to the increased number of organisms in the eight hour culture.

The quantitative aspects of the inoculum effect cannot be investigated by the disk method, therefore, a more refined method of testing the penicillin sensitivity of the organisms was used. This method was a pour plate sensitivity test in which the penicillin was incorporated into the medium used to make the pour plates. By using the pour plate method, it was possible to vary the concentration of the penicillin as well as the age and concentration of the organisms. Table V illustrates the results of the pour plate sensitivity tests.

TABLE V

POUR PLATE SENSITIVITY TESTS

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #1-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	-	-
10-2	TNC	2,700	2	0	-	-
10-4	7,500	25	0	0	-	-
10-6	274	0	0	0	-	-

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #1-b						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	-	-
10-2	TMC	12,540	0	0	-	-
10-3	26,800	85	0	0	-	-
10-4	6,960	4	0	0	-	-
10-6	1,272	2	0	0	-	-
Strain #1-c						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	0	0	0	-
10-2	TNC	TNC	0	0	0	-
10-3	TNC	TNC	0	0	0	-
10-4	TNC	TNC	0	0	0	-
10-6	3,600	3,720	0	0	0	-
Strain #1-d						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	TNC	TNC	1000	-
10-3	TNC	TNC	TNC	0	0	-
10-4	TNC	TNC	11,800	0	0	-
10-6	TNC	3,180	2,400	0	0	-
Strain #1-e						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	TNC	TNC	1	-
10-3	TNC	TNC	27,000	7	0	-
10-4	TNC	20,040	5,400	0	0	-
10-6	23,760	16,200	4,300	0	0	-

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #1-f						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	TNC	TNC	12,200	-
10-3	TNC	TNC	TNC	11,700	63	-
10-4	24,840	17,280	16,740	132	2	-
10-6	944	727	763	0	0	-
Strain #1-g						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	17,500	-	-
10-2	TNC	1,500	0	0	-	-
10-4	5,040	0	0	0	-	-
10-6	672	0	0	0	-	-
Strain #1-h						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	-	-
10-2	TNC	33,840	2	0	-	-
10-3	36,180	1,938	0	0	-	-
10-4	17,400	110	0	0	-	-
10-6	11,760	102	0	0	-	-
Strain #1-i						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	TNC	0	0	-
10-3	TNC	TNC	10,320	0	0	-
10-4	TNC	6,300	3,360	0	0	-
10-6	103	123	15	0	0	-

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #1-j						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	TNC	TNC	TNC	-
10-3	TNC	TNC	TNC	5,820	0	-
10-4	TNC	16,140	8,640	0	0	-
10-6	660	560	340	0	0	-
Strain #1-k						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	TNC	7,320	37	-
10-3	TNC	35,260	103	0	0	-
10-4	17,240	4,920	0	0	0	-
10-6	590	39	0	0	0	-
Strain #1-l						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	26,760	17,280	1,200	-
10-3	TNC	21,220	123	0	0	-
10-4	19,380	3,660	0	0	0	-
10-6	762	155	0	0	0	-
Strain #1-m						
Dil. of 18 hr. cult.						
Until.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	2,640	1	2	0	-
10-3	11,280	528	2	0	0	-
10-4	3,720	10	0	0	0	-
10-6	1,560	2	0	0	0	-

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #1-n						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	-	-
10-2	TNC	41,040	1,580	6	-	-
10-3	27,000	6,300	0	0	-	-
10-4	7,980	1,262	0	0	-	-
10-6	3,300	232	0	0	-	-
Strain #1-o						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	-	-
10-2	TNC	40,000	7	0	-	-
10-3	54,000	2,160	0	0	-	-
10-4	19,020	87	0	0	-	-
10-6	6,490	24	0	0	-	-
Strain #2-a						
Dil. of 18 hr. cult.						
Undil.	TNC	0	0	0	0	-
10-2	TNC	0	0	0	0	-
10-3	22,680	0	0	0	0	-
10-4	5,700	0	0	0	0	-
10-6	442	0	0	0	0	-
Strain #3-a						
Dil. of 18 hr. cult.						
Undil.	TNC	0	0	0	0	-
10-2	TNC	0	0	0	0	-
10-3	TNC	0	0	0	0	-
10-4	4,740	0	0	0	0	-
10-6	361	0	0	0	0	-

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #4-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	TNC	TNC	28,420	-
10-3	21,300	23,760	14,400	6,480	15	-
10-4	6,300	5,400	2,400	3	0	-
10-6	238	247	20	0	0	-
Strain #5-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	-
10-2	TNC	TNC	3,120	0	0	-
10-3	21,600	18,900	0	0	0	-
10-4	8,840	5,400	0	0	0	-
10-6	297	227	0	0	0	-
Strain #6-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	12,960	-
10-2	TNC	24,840	93	0	0	-
10-3	11,880	3,000	0	0	0	-
10-4	1,980	334	0	0	0	-
10-6	79	10	0	0	0	-
Strain #7-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	15,660	-
10-2	TNC	4,470	1	0	0	-
10-3	15,660	258	0	0	0	-
10-4	2,820	12	0	0	0	-
10-6	133	1	0	0	0	-

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #8-a						
Dil. of 18 hr. cult.						
Undil.	TNC	0	0	0	0	-
10-2	TNC	0	0	0	0	-
10-3	21,660	0	0	0	0	-
10-4	7,740	0	0	0	0	-
10-6	672	0	0	0	0	-
Strain #9-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	150	0	0
10-3	TNC	TNC	24	0	0	0
10-4	8,160	9,120	0	0	0	0
10-6	294	314	0	0	0	0
Strain #9-b						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	262	0	0
10-3	TNC	TNC	382	2	0	0
10-4	18,360	15,660	0	0	0	0
10-6	288	329	0	0	0	0
Strain #9-c						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	0	0
10-3	TNC	TNC	TNC	0	0	0
10-4	9,860	10,800	5	0	0	0
10-6	325	285	5	0	0	0

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #9-d						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	13,500	8,640	-	0
10-3	TNC	TNC	2,820	130	-	0
10-4	5,160	5,460	4	0	0	0
10-6	105	208	0	0	0	0
Strain #9-e						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	5,580	65	0	0
10-3	TNC	TNC	0	0	0	0
10-4	5,840	6,120	0	0	0	0
10-6	181	165	0	0	0	0
Strain #10-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	0
10-2	TNC	TNC	15,660	3	0	0
10-3	29,160	27,540	26	0	0	0
10-4	7,680	6,960	2	0	0	0
10-6	193	152	0	0	0	0
Strain #11-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	12,960	3	0
10-3	TNC	TNC	308	0	0	0
10-4	17,820	21,060	0	0	0	0
10-6	458	426	0	0	0	0

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #11-b						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	0	0
10-3	TNC	TNC	286	0	0	0
10-4	28,620	25,380	0	0	0	0
10-6	648	430	0	0	0	0
Strain #11-c						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	0	0
10-3	TNC	TNC	75	0	0	0
10-4	24,840	22,140	0	0	0	0
10-6	556	434	0	0	0	0
Strain #11-d						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	24,840	6	0
10-3	TNC	TNC	392	0	0	0
10-4	21,600	18,360	0	0	0	0
10-6	430	56	0	0	0	0
Strain #11-e						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	278	0
10-3	TNC	TNC	262	0	0	0
10-4	TNC	TNC	0	0	0	0
10-6	1,020	960	0	0	0	0

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #12-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	TNC	0
10-3	TNC	28,080	20,520	4,080	15	0
10-4	7,200	5,400	376	0	0	0
10-6	320	222	0	0	0	0
Strain #12-b						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	TNC	0
10-3	TNC	TNC	31,860	5,460	3	0
10-4	18,900	17,820	1,680	0	0	0
10-6	338	338	0	0	0	0
Strain #12-c						
Dil. of 18 hr. cult.						
Undil.	840	840	376	100	36	0
10-2	28	19	0	0	0	0
10-3	13	4	0	0	0	0
10-4	3	0	0	0	0	0
10-6	0	0	0	0	0	0
Strain #13-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	TNC	1,260
10-3	TNC	TNC	TNC	TNC	1,980	0
10-4	TNC	TNC	3,000	28	0	0
10-6	1,460	1,260	4	0	0	0

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #14-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	12,900	62	1
10-3	TNC	TNC	6,780	373	0	0
10-4	19,980	7,520	135	0	0	0
10-6	368	320	0	0	0	0
Strain #14-b						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	12	0
10-3	TNC	17,820	3,720	123	0	0
10-4	7,560	12,960	301	0	0	0
10-6	368	400	4	0	0	0
Strain #14-c						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	29,160	0	0
10-3	TNC	31,320	5,520	49	0	0
10-4	TNC	19,980	138	0	0	0
10-6	508	562	0	0	0	0
Strain #15-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	TNC	420
10-3	TNC	TNC	TNC	TNC	190	0
10-4	TNC	TNC	4,560	14	0	0
10-6	1,184	836	0	0	0	0

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #16-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	4,020	0	0
10-3	TNC	TNC	743	0	0	0
10-4	TNC	TNC	0	0	0	0
10-6	634	609	0	0	0	0
Strain #17-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	20	0
10-3	TNC	TNC	TNC	150	0	0
10-4	TNC	7,560	2	0	0	0
10-6	348	416	0	0	0	0
Strain #18-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	0
10-2	TNC	0	0	0	0	0
10-3	TNC	0	0	0	0	0
10-4	1,380	0	0	0	0	0
10-6	30	0	0	0	0	0
Strain #18-b						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	0
10-2	TNC	0	0	0	0	0
10-3	TNC	0	0	0	0	0
10-4	900	0	0	0	0	0
10-6	21	0	0	0	0	0

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #19-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	TNC	66
10-3	TNC	TNC	TNC	1,650	0	0
10-4	TNC	TNC	0	0	0	0
10-6	1,440	900	0	0	0	0
Strain #20-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	0	0
10-3	TNC	TNC	0	0	0	0
10-4	TNC	2,700	0	0	0	0
10-6	1,080	58	0	0	0	0
Strain #20-b						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	TNC	0
10-3	TNC	TNC	TNC	450	0	0
10-4	TNC	TNC	0	0	0	0
10-6	440	75	0	0	0	0
Strain #20-c						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	46	0
10-3	TNC	TNC	22	0	0	0
10-4	TNC	TNC	0	0	0	0
10-6	103	96	0	0	0	0

TABLE V (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #20-d						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	0	0
10-3	TNC	TNC	0	0	0	0
10-4	TNC	1,700	0	0	0	0
10-6	108	58	0	0	0	0
Strain #20-e						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	TNC	0	0
10-3	TNC	TNC	TNC	15	0	0
10-4	TNC	756	0	0	0	0
10-6	34	41	0	0	0	0
Strain #21-a						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	14	0	0
10-3	TNC	TNC	0	0	0	0
10-4	TNC	448	0	0	0	0
10-6	885	5	0	0	0	0

The possibility of a mixed culture was considered when it was noticed that there were some very resistant colonies growing in the presence of 100 units of penicillin. To

further investigate this, the following experiment was performed: An 18 hour broth culture of a colony resistant to 100 units of penicillin was reinoculated into nutrient broth and incubated for 18 hours at 37°C. This culture was then subjected to the same pour plate sensitivity test as the parent strain. No difference was detected in the penicillin susceptibility of the culture when compared to the parent strain.

The following experiment was designed to determine if penicillin resistant organisms could destroy or bind penicillin, thus allowing a penicillin sensitive organism to grow. By using the ditch method of testing for penicillin sensitivity, a sensitive strain of alpha hemolytic streptococci was chosen as the penicillin sensitive test organism. This organism was streaked perpendicular to the penicillin ditch. A resistant staphylococci was also streaked in a like manner but on both sides of the streptococci. It was felt that if the staphylococci could destroy the penicillin then the streptococci could grow closer to the ditch of penicillin than was previously possible. These results indicated that the staphylococci did not destroy or bind the penicillin either completely enough or soon enough to prevent the destruction of the streptococci by the penicillin.

Sodium azide, being an enzyme poison, will suppress the production of penicillinase (Bondi et al., 1948b).

Because of its action sodium azide was incorporated into the growth medium. The normal pour plate assay of penicillin sensitivity was run, using strain #11-d as the organism being tested. The control for this experiment consisted of a normal nutrient broth culture subjected to no sodium azide neither in the broth nor in the agar used for the pour plate test. Table VI contains the results of this experiment.

TABLE VI

EFFECT OF SODIUM AZIDE ON PENICILLIN RESISTANCE

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Control: No azide in agar or broth						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	2,100	1	0
10-3	17,280	15,660	1,080	0	0	0
10-4	3,440	3,360	51	0	0	0
10-6	60	70	0	0	0	0
No azide in the agar; 0.01% azide in the nutrient broth						
Dil. of 18 hr. cult.						
Undil.	27	22	21	20	13	10
10-2	3	5	8	3	10	7
10-3	2	9	11	3	4	3
10-4	1	1	6	0	4	0
10-6	7	5	3	0	0	2

TABLE VI (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
No azide in the agar; 0.1% azide in the nutrient broth						
Dil. of 18 hr. cult.						
Undil.	165	139	120	59	36	132
10-2	4	24	10	3	5	3
10-3	9	5	4	2	5	4
10-4	12	13	0	1	1	1
10-6	25	10	2	0	1	2
No azide in the nutrient broth; 0.01% azide in the agar						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	TNC	TNC	TNC	TNC
10-2	TNC	TNC	TNC	876	13	0
10-3	15,660	20,840	346	0	0	0
10-4	3,360	2,940	15	0	0	0
10-6	79	54	5	0	0	0
No azide in the nutrient broth; 0.1% azide in the agar						
Dil. of 18 hr. cult.						
Undil.	24	202	197	8	6	4
10-2	0	0	0	0	0	0
10-3	1	0	0	0	0	0
10-4	0	0	0	0	0	0
10-6	0	0	0	0	0	0
0.01% azide in both agar and nutrient broth						
Dil. of 18 hr. cult.						
Undil.	131	66	103	82	47	82
10-2	3	0	0	0	0	1
10-3	0	0	2	0	1	0
10-4	0	0	2	0	0	0
10-6	0	0	0	0	0	0

TABLE VI (continued)

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
0.1% azide in both agar and nutrient broth						
Dil. of 18 hr. cult.						
Undil.	12	7	10	7	1	5
10-2	3	6	1	1	0	0
10-3	2	0	0	0	1	0
10-4	2	1	2	0	1	0
10-6	2	0	4	0	2	0

From the above results (Table VI), it seemed that when the azide was added to the broth, the cells possibly became more resistant to penicillin.

From the results of the routine pour plate sensitivity tests (Table V), it was decided that the sensitivity of the method could be improved by changing the penicillin concentrations and number of organisms in the inoculum. The pour plate method was redesigned with penicillin concentrations of 1, 5, 10, 25, 50, 75, 100, and 1000 unit(s) being used. Accompanying this change, the concentration of organisms was altered and, for an inoculum, a series of nine two-fold dilutions of a broth culture were obtained. The experiment was run in the normal manner and the results obtained by

counting the colonies on the pour plates. The organism being tested in this case was the strain #11-d. The results of this experiment are presented in Table VII.

As can be seen from these results (Table VII), a growth or no growth phenomenon resulted. When the log of the inhibitory concentration of penicillin is plotted against the log of the inoculum size a direct relationship can be seen as is shown in Figure 2, page 60.

Further insight concerning the mechanisms of penicillin resistance was obtained by the use of combined broth and pour plate methods as previously described.

The results of the first experiment of this type illustrated the need to inactivate the penicillin with penicillinase before the pour plates were prepared. If this was not done, the pour plates did not give reliable results. Also, from the results of this experiment, it was determined that a standard inoculum must be used. For this reason, a transmittance curve of the strain #11-d was made noting the number of organisms and the per cent transmittance of the culture. Figure 3, page 61, illustrates the results of this curve. From this curve, it is possible to obtain an inoculum of any given bacterial concentration.

This combination tube and pour plate sensitivity test was carried out in a manner similar to the previous experiments in that 50 units of penicillin per ml of broth was used.

TABLE VII

MODIFIED POUR PLATE SENSITIVITY TEST

Organisms per ml	Units of Penicillin per ml								
	1U.	5U.	10U.	25U.	50U.	75U.	100U.	500U.	1000U.
6.0×10^8	TNC	TNC	TNC	TNC	TNC	TNC	TNC	TNC	TNC
3.0×10^8	TNC	TNC	TNC	TNC	TNC	TNC	TNC	TNC	0
1.8×10^8	TNC	TNC	TNC	TNC	TNC	TNC	TNC	0	0
7.5×10^7	TNC	TNC	TNC	TNC	TNC	TNC	TNC	0	0
3.75×10^7	TNC	TNC	TNC	TNC	TNC	0	0	0	0
1.9×10^7	TNC	TNC	TNC	0	0	0	0	0	0
9.4×10^6	TNC	TNC	0	0	0	0	0	0	0
4.7×10^6	TNC	0	0	0	0	0	0	0	0
2.3×10^6	TNC	0	0	0	0	0	0	0	0
1.2×10^6	0	0	0	0	0	0	0	0	0

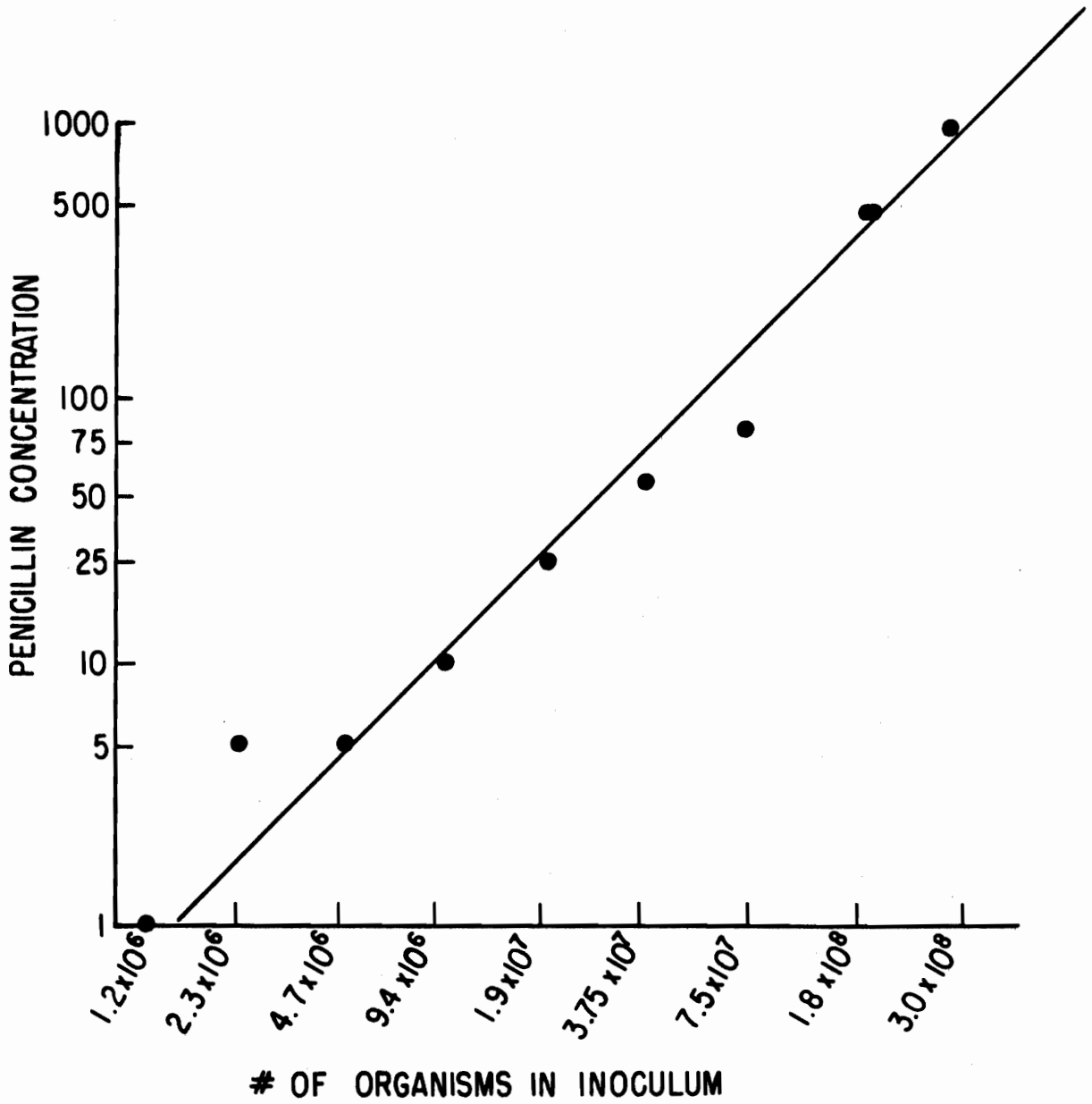

Figure 2. Effect of inoculum size on penicillin resistance.

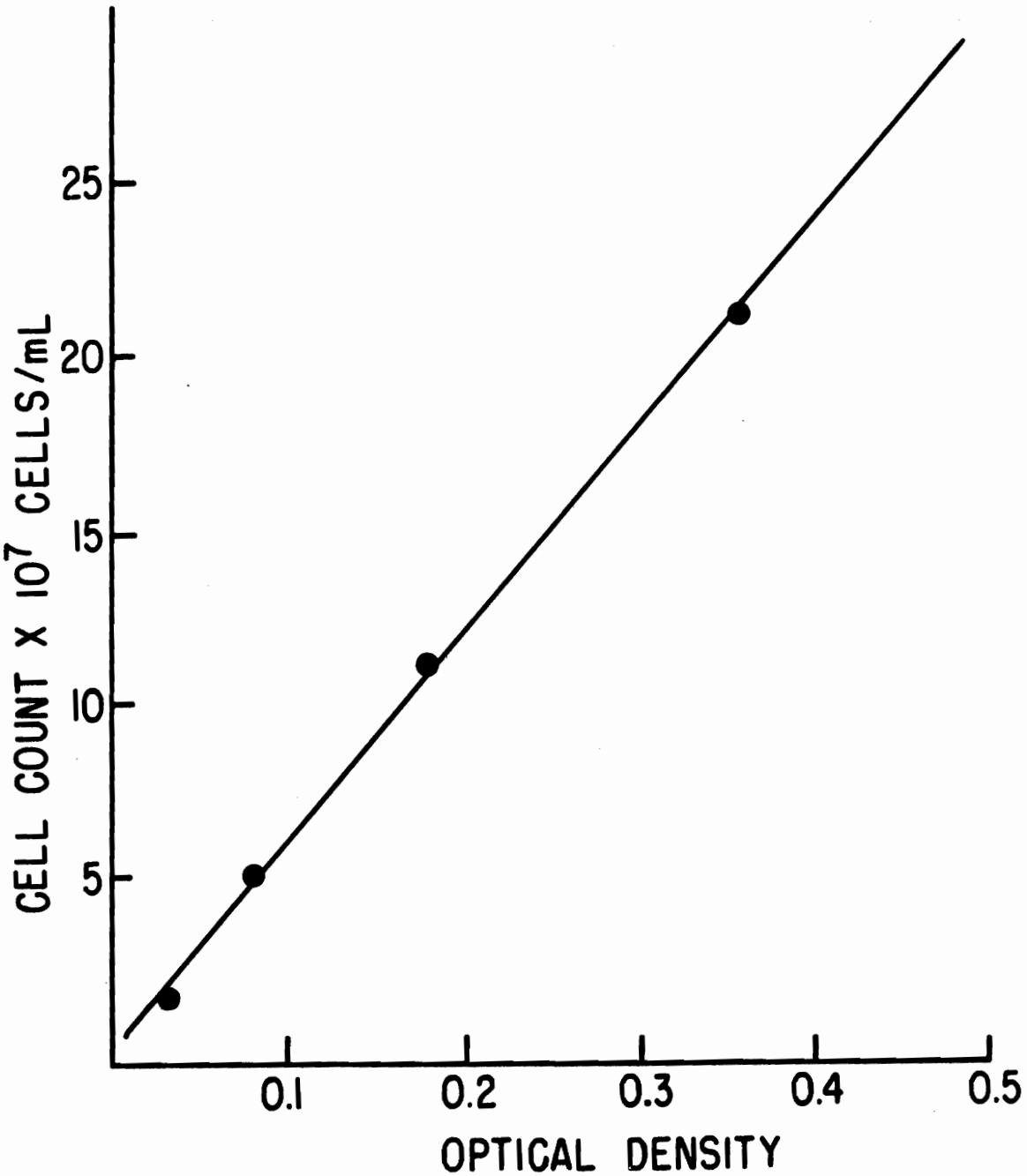

Figure 3. Optical Density curve

To the tubes of broth containing this concentration of penicillin the various numbers of organisms were added. Samples were withdrawn from these tubes and the number of viable organisms determined at 2 hours, 4 hours, 9 hours, 22 hours, and five days. These results are plotted in Figure 4.

From Figure 4, it will be noticed that the growth in the control tube had no appreciable lag after transfer from the original broth. The growth in the first tube containing an inoculum of approximately 3×10^7 cells showed a slight lag between two and four hours, but it is probably of little significance. This growth in tube #1 at nine hours had equaled the growth in the control tube. Tube #2 showed a somewhat different picture. This tube had an inoculum of approximately 15×10^6 cells. At the end of the first two hours, there was a small increase in cell numbers but this decreased to approximately that of the inoculum at four hours. A very slight increase was noticed in cell numbers up until nine hours at which time the culture proceeds at a rate similar to that of the control tube. Tube #3 shows a similar picture; the only difference being that the latent or static period of the cells extends until the twenty-second hour. The same was true for the fourth tube, the case here being that the latent or static period extends until the fourth or fifth day.

The previous experiments indicated a relationship

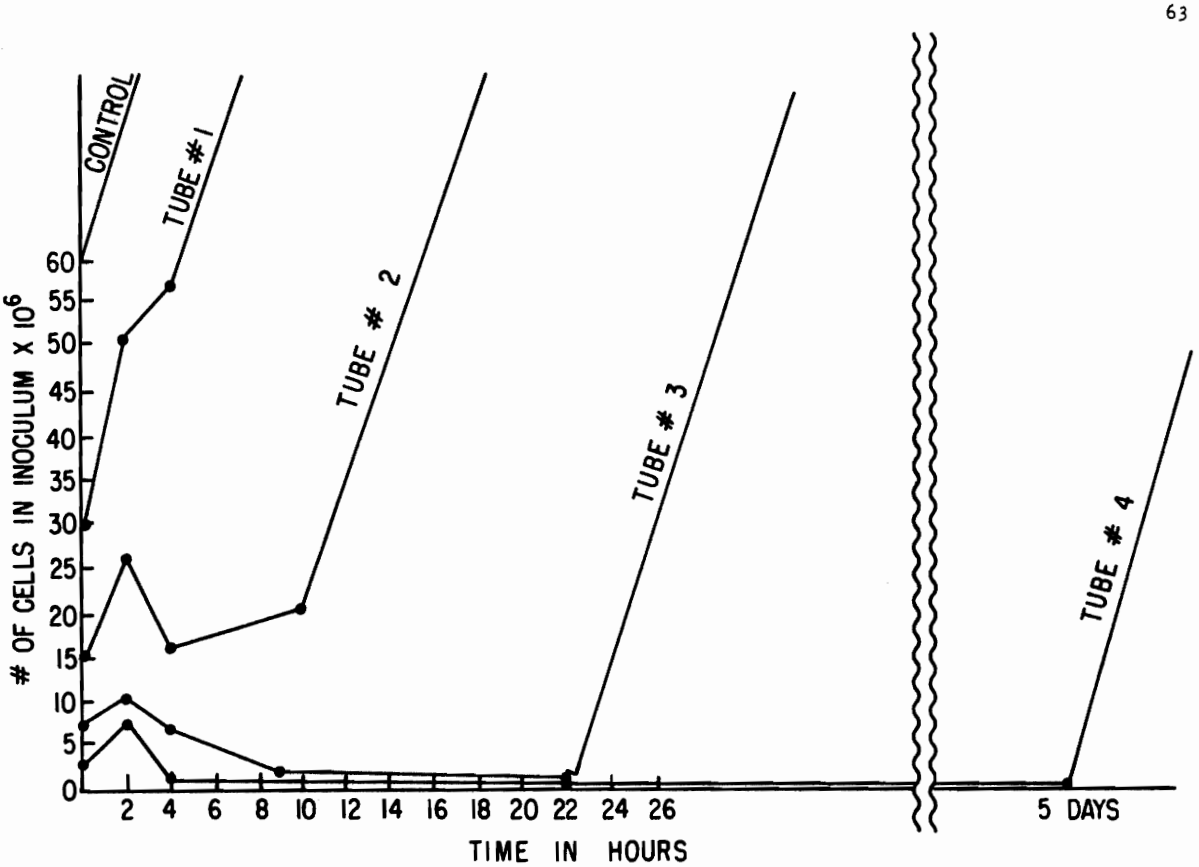

Figure 4. Pour plate sensitivity test.

between the size of the inoculum and penicillin resistance, therefore, it seemed necessary to determine the amount of penicillin remaining in the tubes. The original method of assay for penicillin was a chemical method using an iodometric titration procedure. At first there were great expectations of this procedure, but after several determinations using this method of assay, it was decided that the method worked very well in the higher ranges of penicillin concentration but not for small amounts which would be encountered in this investigation. For this reason the more sensitive bioassay method was used.

The biological assay was a cylinder plate method. Preliminary assays were run and the results were well within the range of expected experimental error. Upon assaying penicillin concentrations of 50 units/ml, it was experimentally determined that this method would give results with an error of + or - 0.9 per cent. The only problem associated with this method was estimating the penicillin concentration in the unknown.

When assaying for penicillin a solution containing staphylococci, inactivation of the penicillinase produced by these organisms was necessary. If the penicillinase was not inactivated, the penicillinase would react with the penicillin in the fluid being assayed and zones would not be seen or possibly a zone of reduced size would result. Penicillinase

is heat labile, therefore, the enzyme was inactivated as follows: The medium containing the penicillin, penicillinase, and staphylococci was boiled for ten minutes and then assayed for penicillin. When compared with the standard, there was no measurable amount of penicillin degradation due to the boiling.

With the preliminary experiments accomplished, an experiment was designed to further investigate the role of the residual penicillin. The design of this experiment is presented in Table VIII.

TABLE VIII

EXPERIMENTAL DESIGN; COMBINATION TUBE AND POUR PLATE SENSITIVITY TESTS PLUS PENICILLIN ASSAY

Tube No.	Number of Cells per ml
1	7×10^6
2	14×10^6 50 units of penicillin/ml
3	7×10^6 50 units of penicillin/ml
4	50 units of penicillin/ml

After incubation at 37°C, samples were taken from the tubes at 2 hours, 6 hours, 12 hours, and 24 hours. At each time pour plates were made from the tubes containing the inoculum of organisms. Penicillin determinations were made on aliquots taken from the tubes containing penicillin. The results are shown in Figure 5. From this graph, it is seen that penicillin determinations were not included past the 2 hour time period. The penicillin concentration at this time dropped below that concentration estimated and the assay results were in a concentration range in which a greater experimental error could result. The experiment demonstrated that the penicillin concentration dropped rapidly into such a range that the staphylococci were no longer inhibited by the penicillin.

With the results of the previous test (Figure 5), there arose the problem of detecting the mechanism for the rapid decrease in penicillin concentration. The control of this experiment indicated that the penicillin was not being inactivated at a rapid rate because of the direct effect of the incubator temperature, nor was it due to chemical components of the broth. This left one of two possibilities, or a combination of both: (1) The staphylococci could be binding the penicillin and thus lowering the concentration in the broth, or, (2) the staphylococcal penicillinase was acting on the penicillin and thus inactivating it.

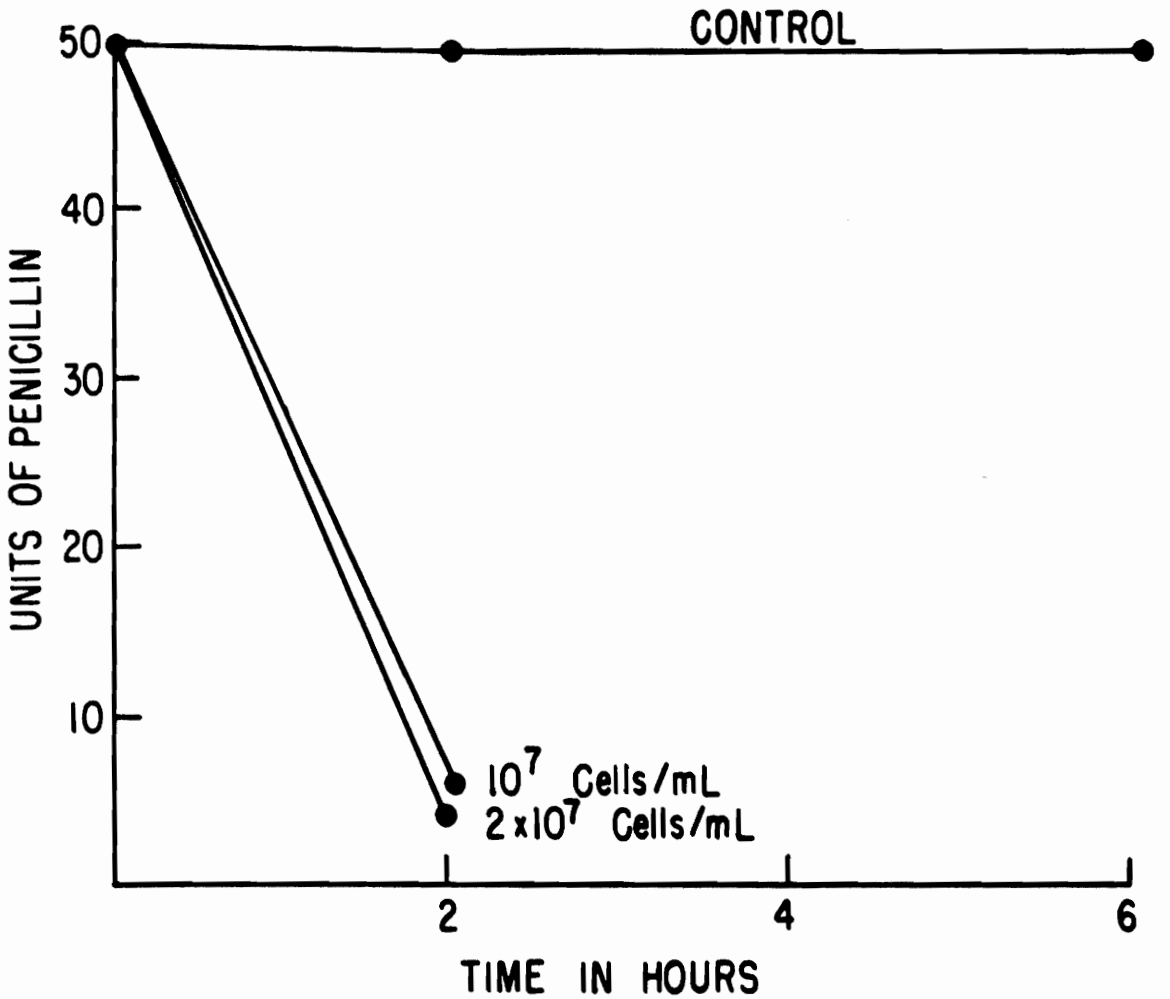

Figure 5. Penicillin assay, tube and pour plate test.

To determine the effect of binding of the penicillin by the staphylococcal cells, the following experimental design was used: Staphylococcal cells were washed and re-suspended in sterile saline in a concentration of approximately 10^4 and 10^6 cells per ml. Penicillin was added to the cell suspensions to give a final concentration of 50 units of penicillin per ml. A biological assay was run at the beginning of the experiment and the tubes placed in a refrigerator at 4°C. After 30 minutes the tubes were removed and centrifuged at 20,000 G for 30 minutes to sediment the bacterial cells. An aliquot of the supernatant fluid was removed and assayed for penicillin activity. By incubating the cells in the refrigerator, the action of the penicillinase on the penicillin should be negligable, due to the inactivity of penicillinase at this temperature. The procedure was repeated at one hour and the results obtained are shown in Figure 6. Figure 6 indicates that the total binding of the penicillin to the staphylococcal cells is independent of the number of cells. One cell will bind only a certain amount of penicillin. These results suggest that the binding of the penicillin to the staphylococcal cells might account for the approximately ten of the fifty original units of penicillin added to the cell suspension. This experiment also suggests that the inactivation of the penicillin by heat for the first few hours is very slight. The penicillin control tube assayed

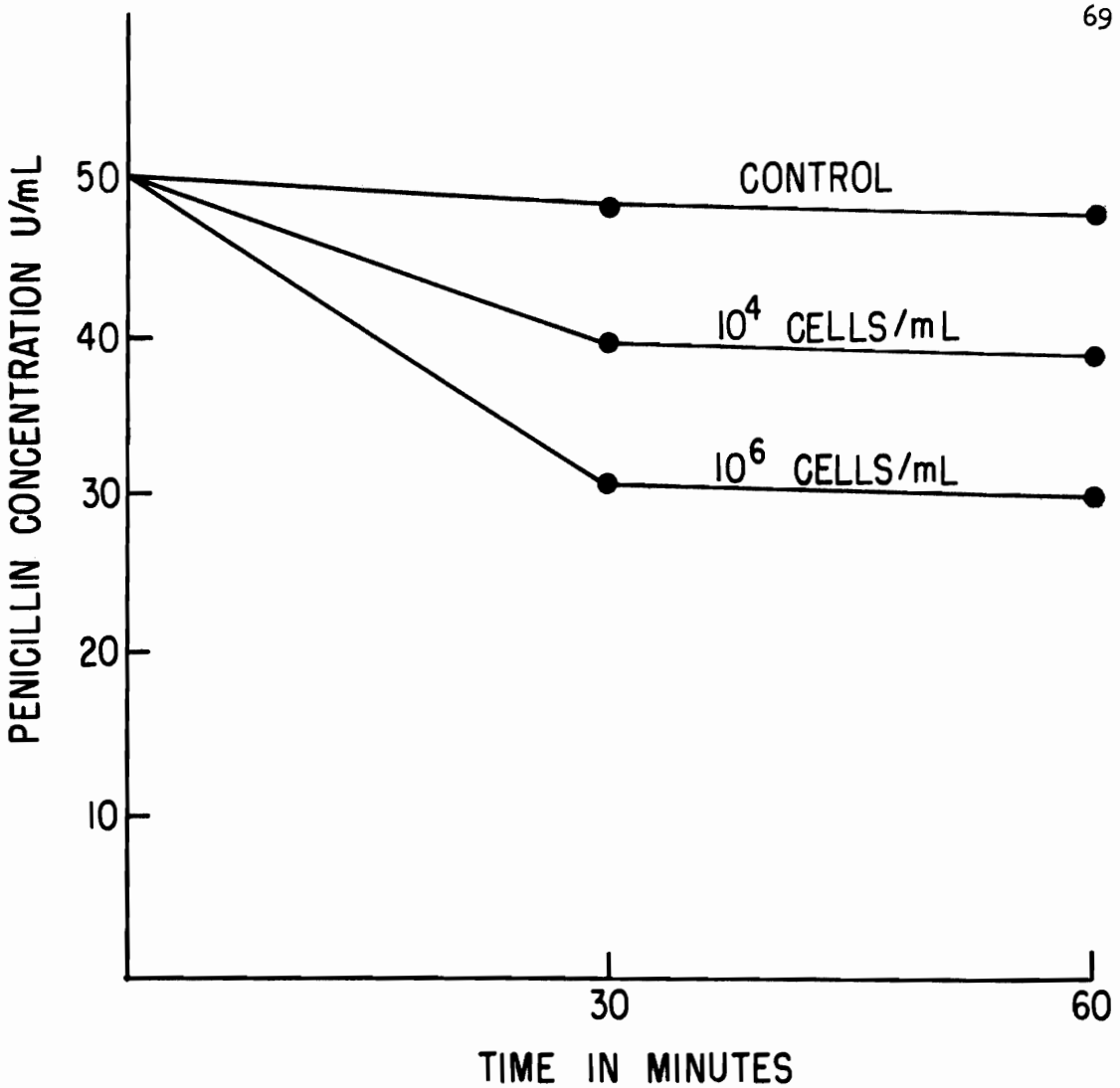

Figure 6. Adsorption of penicillin by staphylococcal cells.

after standing in the refrigerator lost about the same amount of potency as the control tube incubated at 37°C.

A low concentration of penicillin was also employed to determine whether the bacterial suspension would bind the entire amount of penicillin present. The results obtained in this experiment are illustrated in Figure 7. It is seen from these results that the higher the concentration of organisms, the greater the total binding power. It is also of importance to note that the lower concentration of penicillin was completely bound by the greater number of organisms, and nearly bound by the lower number of organisms.

A study designed to determine the difference in the binding power between a penicillin sensitive and a penicillin resistant strain of staphylococci did not give conclusive results. The difference in binding power, if any, was too small to detect by the method of assay used in this study. There is some suggestion, however, that the sensitive strains bind more penicillin than the resistant strains, but definite results were not obtained.

Figure 8 is a photograph of the typical assay plates obtained in the course of assaying for penicillin by the method used in this study. The bottom center well contained the 1.0 unit/ml standard; the upper center well contained the 0.25 unit standard. The left well contained the estimated 1.0 unit/ml dilution of the unknown sample, and the right

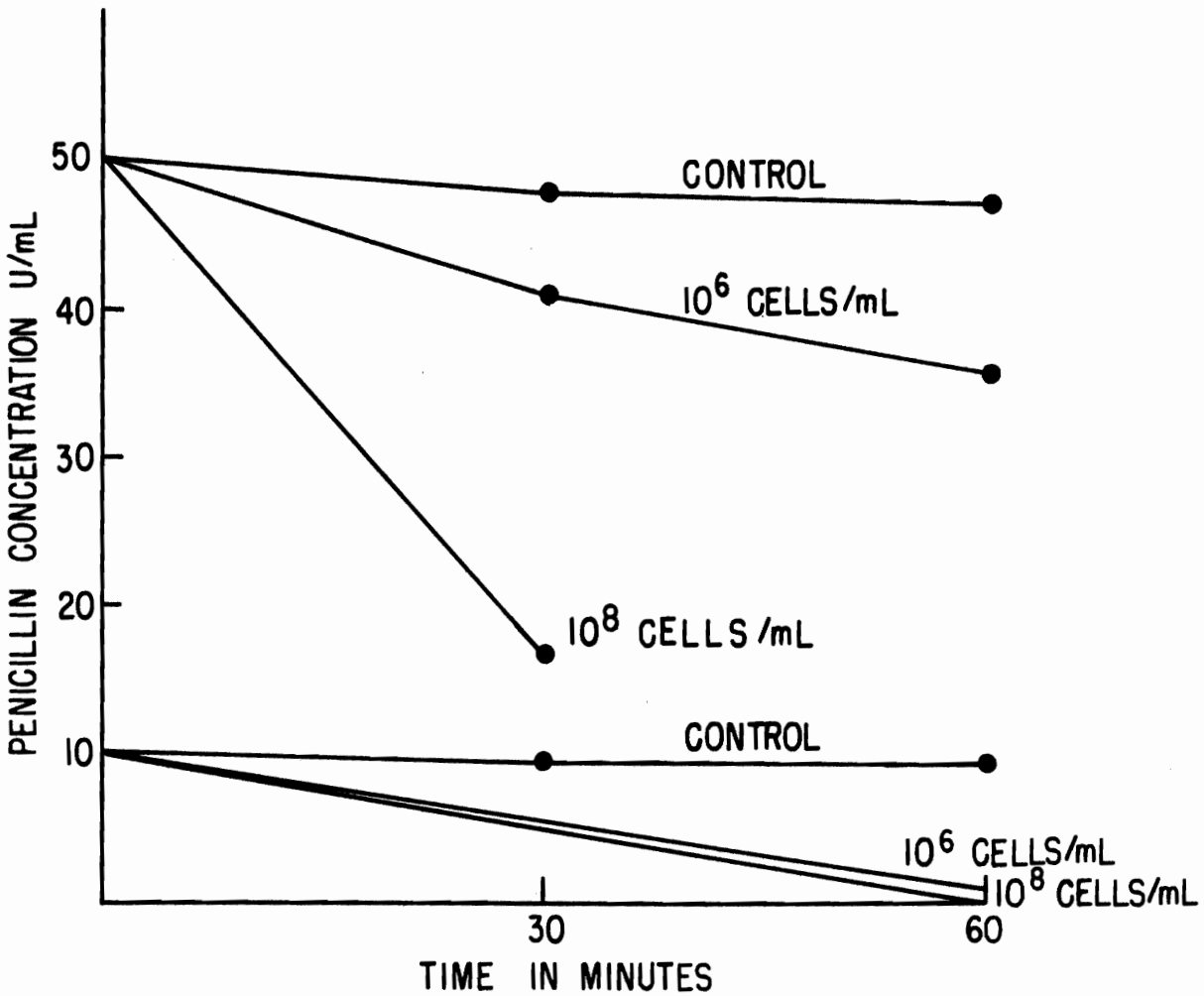

Figure 7. Effect on adsorption by varying concentrations of penicillin and organisms.

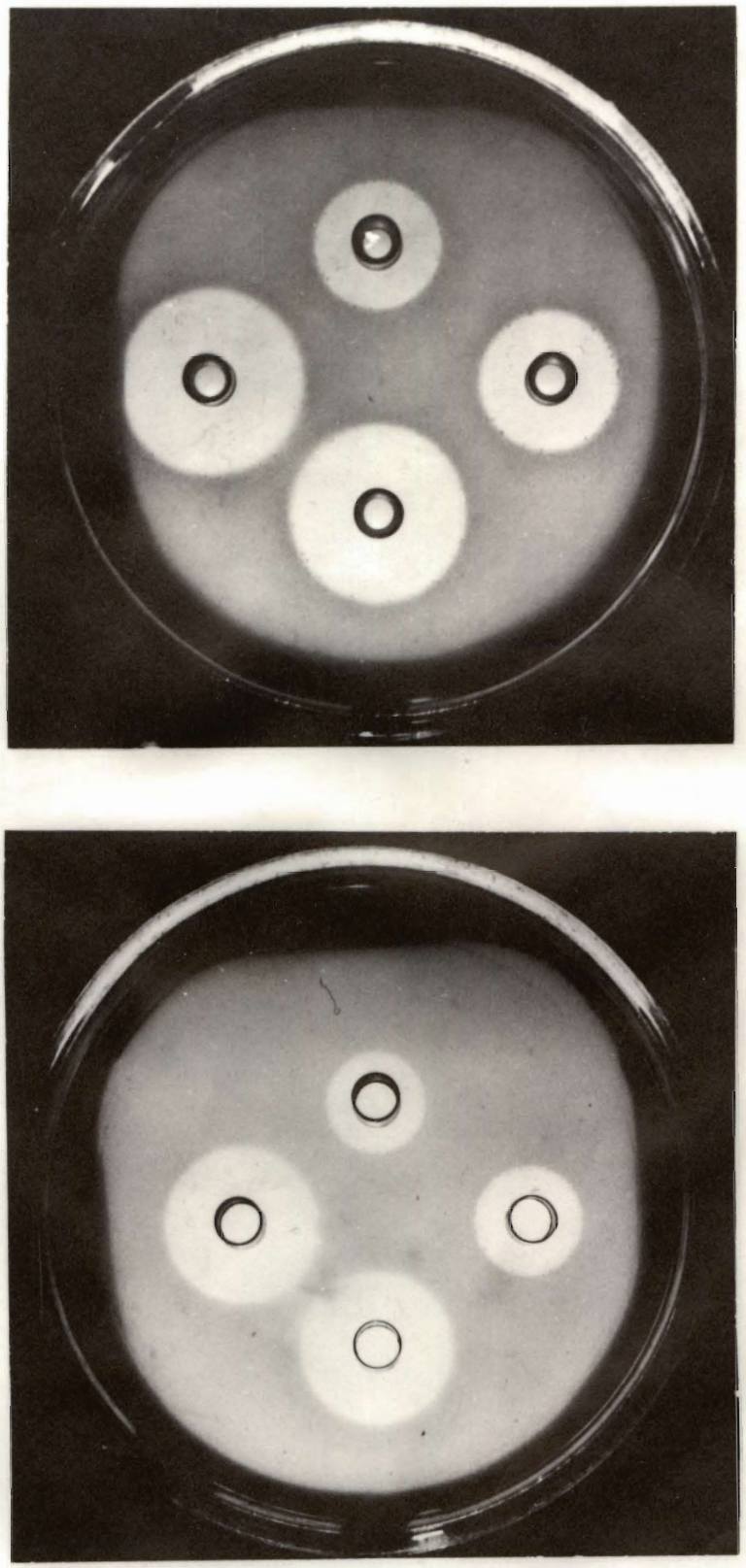

Figure 8. Photograph of assay plates

well contained the estimated 0.25 unit/ml dilution of the sample.

This method of assay may be adapted to measurement of penicillin concentrations as low as 0.005 units per ml and although quite laborious, the results are very reliable.

The effect of Ca^{++} and Mg^{++} on the adsorption of penicillin to the bacterial cell was studied. In this experiment, 0.005 M and 0.05 M concentrations of each ion were used. This experiment was carried out in the same manner as the previous experiments on penicillin adsorption with the exception that the penicillin solution was made up in the above ion solutions. The reactant solutions were incubated and assayed for penicillin in the same manner as the previous experiments. The results of this experiment are found in Figure 9. The results were inconclusive, however, a comparison of these results to a previous experiment suggest that there might be a competitive or inhibitive action by the Ca^{++} and Mg^{++}. This could result in higher concentrations of the free penicillin when the ions are present. If the ions hastened or aided in the attachment of the penicillin, one could expect to find a lower concentration of penicillin in the supernatant fluid.

During the recent months dimethoxyphenyl penicillin was marketed. This penicillin is a chemical variant of the biologically produced penicillin G. Dimethoxyphenyl

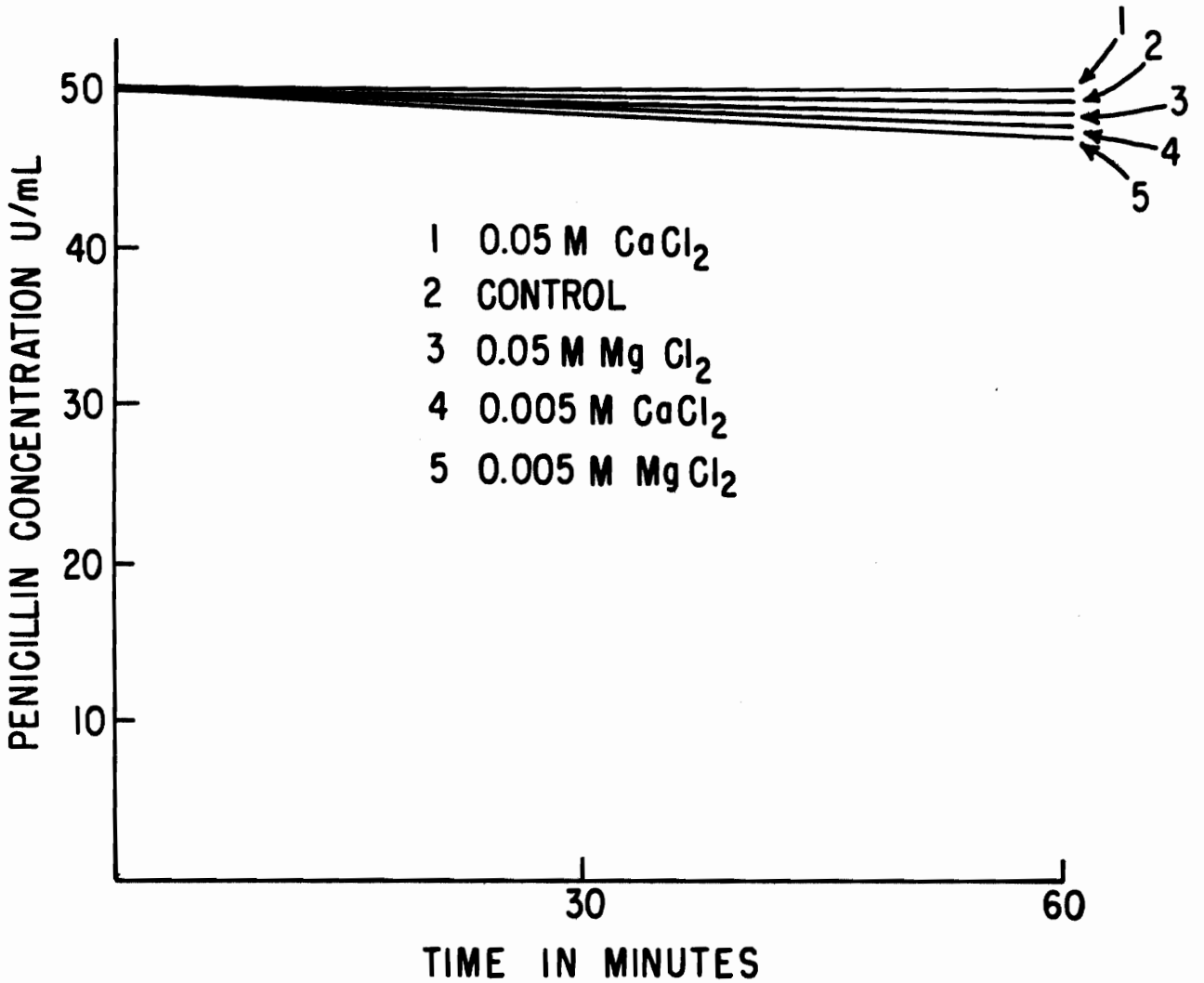

Figure 9. Effect of Mg++and Ca++on penicillin adsorption by staphylococcal cells.

penicillin was reported to have bactericidal action against the penicillin resistant staphylococci. Because of the claim, a small amount of this chemical was obtained from the Bristol Laboratories. The dimethoxyphenyl penicillin received was a portion of the lot #10cd32. Along with this penicillin, some experimental information about this new drug was received. From this information the following results, illustrated in Figures 10 and 11, are taken. These figures are self-explanatory and illustrate the resistance of dimethoxyphenyl penicillin to the action of staphylococcal penicillinase.

Table IX illustrates the results obtained when the strain #11-d was used as an inoculum for the pour plate sensitivity test using dimethoxyphenyl penicillin in place of penicillin G.

TABLE IX

POUR PLATE SENSITIVITY TESTS USING DIMETHOXYPHENYL PENICILLIN

	Units of Penicillin per ml					
	0.0	0.1	1.0	10	100	1000
Strain #11-d						
Dil. of 18 hr. cult.						
Undil.	TNC	TNC	2	0	0	0
10-2	TNC	TNC	0	0	0	0
10-3	TNC	TNC	4	0	0	0
10-4	TNC	TNC	9	0	0	0
10-6	428	322	186	0	0	0

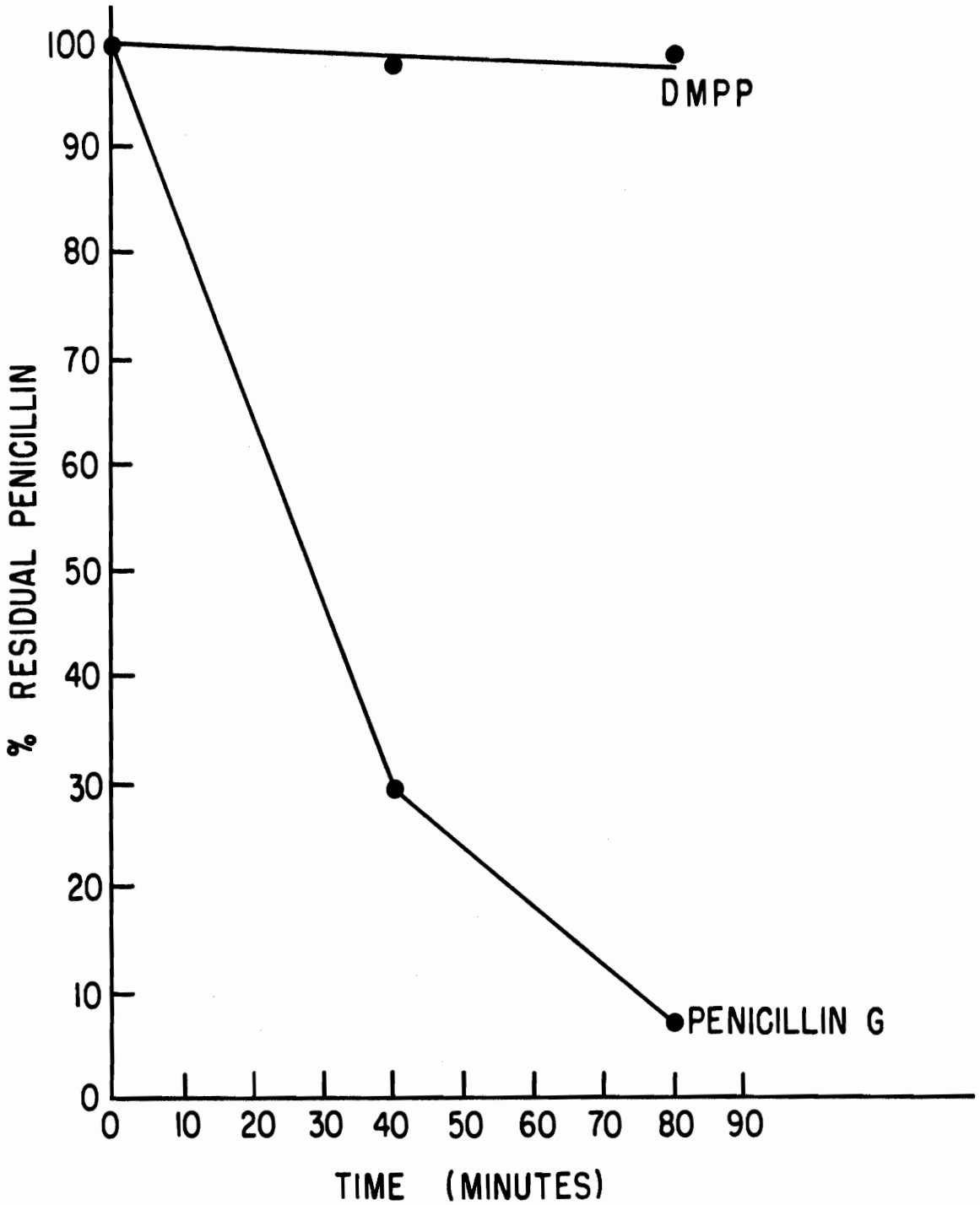

Figure 10. Action of penicillinase on demethoxyphenyl penicillin and penicillin G.

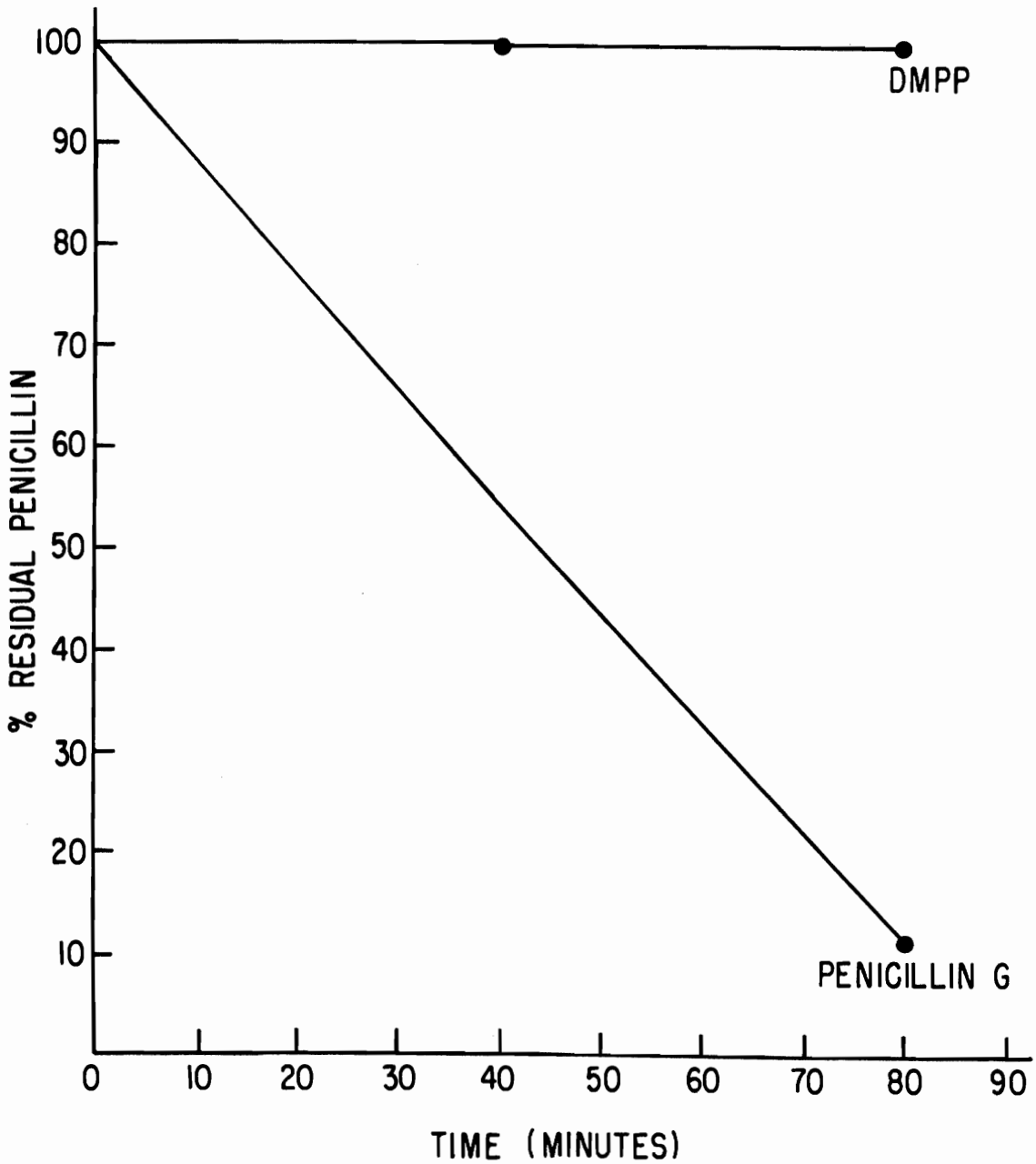

Figure 11. Action of cell free penicillinase on dimethoxyphenyl penicillin and penicillin G.

The above concentrations of dimethoxyphenyl penicillin were made on weight basis. The above unitage of this drug is based on that of penicillin G which is 60 mg = 1,000,000 units. By using this concentration of dimethoxyphenyl penicillin, the pour plate sensitivities on both this drug and the Penicillin G have the same weight per given unitage. As can be seen by the previous results, the dimethoxyphenyl penicillin had a marked inhibitory effect on this strain of staphylococcus. Partial inhibition occurred at one unit but complete inhibition occurred at the 10 unit level. With the reported resistance of this penicillin to staphylococcal penicillinase, it seemed probable that the penicillinase might be the causative agent behind the inoculum effect.

Working on the theory that possible binding of staphylococcal penicillinase could occur when dimethoxyphenyl penicillin is used, a study was designed to determine the combined effect of dimethoxyphenyl penicillin and penicillin G.

An inoculum of 1 ml of a 10^{-2} dilution of an 18 hour broth culture was used per plate. This amounted to 27×10^7 cells. Varying concentrations of penicillin G and dimethoxyphenyl penicillin were used in the agar layer of these pour plates. The concentration of these antibiotics can be found in Table X. The first number in each block is the concentration of dimethoxyphenyl penicillin; the second is that of the

penicillin G. The lower number is the number of colonies present in that pour plate at the end of 24 hours incubation at 37°C.

TABLE X

COMBINED EFFECT OF POUR PLATE SENSITIVITIES OF PENICILLIN G AND DIMETHOXYPHENYL PENICILLIN

Concentration of Dimethoxyphenyl Penicillin-- Concentration of Penicillin G						
Colonies Present in Pour Plate						
0-0	0-3.1	0-6.3	0-12.5	0-25	0-50	0-100
TNC	197	53	9	10	3	3
0.16-0	0.16-3.1	0.16-6.3	0.16-12.5	0.16-25	0.16-50	0.16-100
TNC	230	140	7	8	2	2
0.31-0	0.31-3.1	0.31-6.3	0.31-12.5	0.31-25	0.31-50	0.31-100
TNC	310	50	13	7	2	2
0.63-0	0.63-3.1	0.63-6.3	0.63-12.5	0.63-25	0.63-50	0.63-100
TNC	130	44	25	7	3	1
1.3-0	1.3-3.1	1.3-6.3	1.3-12.5	1.3-25	1.3-50	1.3-100
TNC	44	35	9	5	0	2
2.5-0	2.5-3.1	2.5-6.3	2.5-12.5	2.5-25	2.5-50	2.5-100
TNC	3	0	0	1	0	0
5-0	5-3.1	5-6.3	5-12.5	5-25	5-50	5-100
0	0	0	0	0	0	0
10-0	10-3.1	10-6.3	10-12-5	10-25	10-50	10-100
0	0	0	0	0	0	0

There appeared to be some combined effect of the Dimethoxyphenyl penicillin and the penicillin G at the 2.5-3.1 level of concentration. There might have been some effect of the two antibiotics in the range of 1.2-50.

DISCUSSION

The staphylococcal strains collected for use in this study were obtained from blood cultures and were assumed to be pathogenic. The only selective requirement was that the strains came from a human infection. The reason for the high incidence of strains producing the inoculum effect is not known, unless penicillinase producing strains cause more infections of the type from which these strains were obtained.

The lack of these strains to fall into a recognizable group when bacteriophage typed can be explained in two ways: (1) A grouping is not possible by comparing phage type and the ability of a strain to produce the inoculum effect, and (2) a study this small being limited in number of strains obtained, a site of infection from which strains were obtained, and geographical location of hospitals participating in this study does not give a true representative sample of the true strain population of staphylococci. The fact that in every case the different isolates of each strain had a similar phage type to the original isolate gives some indication that the same strain was being tested independent of which isolation of that strain was used.

The disk method of penicillin sensitivity testing, while being the easiest method of detecting the inoculum effect, by no means gives the most information as to

concentration of penicillin and number of organisms present in the test. The pour plate method of testing for penicillin sensitivity will give information as to the concentration of penicillin and the number of organisms. When this method is used and a resistant colony is obtained, the common interpretation is that the population of this colony is capable of growing and multiplying in that concentration of penicillin. In actual conditions, when the resistant colony is rerun through the pour plate sensitivity test, it does not differ from the original parent strain in penicillin sensitivity. These results lead one to believe that a mixed culture does not exist.

The fact that sodium azide will inhibit the production of penicillinase gives some hope that an explanation as to the relationship between penicillinase and the inoculum effect might be gained. It was found that the sodium azide, in the concentrations used, either exhibited a marked effect on the cell, not only its penicillinase production but also its necessary metabolic pathways, or exhibited no effect at all.

From the modified pour plate sensitivity test a direct relationship between the number of organisms in the inoculum and the resistance to penicillin was determined.

Other methods of studying this effect were employed. The combination tube and pour plate sensitivity test

illustrated that the concentration of penicillin does not appear to be lethal to all of the cells in the inoculum. This concentration of penicillin appears instead to be bacteriostatic. When the penicillin assay was combined with the combination tube and pour plate test the rate of destruction of the penicillin was apparent. This combined with the adsorption of the penicillin to the cells could explain much of the inoculum effect.

A possible explanation of the static period seen in the combination tube and pour plate method is that the cells in the inoculum are not killed but remain in a state in which metabolic processes are still going on with no division taking place. A product of this metabolism could be penicillinase. When this penicillinase concentration gets high enough the penicillin in the surrounding medium is inactivated. At a time when this penicillin concentration gets below a threshold concentration which is bacteriostatic for the organism's growth, then reproduction results. To support this possible importance of penicillinase in the inoculum effect, experiments were run using Dimethoxyphenyl penicillin. This penicillin is reported to be resistant to the action of penicillinase. When the pour plate method for testing penicillin resistance was run using Dimethoxyphenyl penicillin the inoculum effect was not observed. This is a possible explanation of the importance of penicillinase

production by organisms producing the inoculum effect. The combination of adsorption of penicillin to the bacterial cell and the destruction of the penicillin by penicillinase lowers the concentration of penicillin in the medium from an inhibitory one to a sub-inhibitory one.

SUMMARY

1. Staphylococcus aureus strains have been obtained from blood cultures and kept for further study.

2. All Staphylococcus aureus strains have been bacteriophage typed with no common grouping noticable.

3. Disk sensitivity testing methods have been used to demonstrate the inoculum effect. Importance to clinical application has been mentioned.

4. A pour plate method of testing for penicillin sensitivity has been described and results of strains tested recorded.

5. An attempt was made to demonstrate a mixed culture of cells with a varying sensitivity to penicillin within a given strain. No appreciable difference in penicillin sensitivity could be demonstrated.

6. A ditch method of penicillin sensitivity testing was used to attempt to demonstrate the protection of a sensitive organism from the action of penicillin by a resistant staphylococcus. No protection could be detected.

7. Sodium azide was incorporated in various concentrations into the culture medium. The azide had an effect on the cell reproduction but results were not obtained in relation to the inoculum effect.

8. A modified pour plate method demonstrated a direct relationship between number of organisms in the

inoculum and the degree of resistance of an individual strain of Staphylococcus aureus.

9. Combination tube and pour plate methods of penicillin sensitivity testing demonstrated a stasis period after which growth occurs at a normal rate. The length of static period varies indirectly with the number of organisms in the inoculum.

10. Penicillin assays were carried out by using a chemical method. This method was discarded because of its insensitivity in the lower penicillin concentrations.

11. A biological assay method for penicillin was used to assay for remaining penicillin after incubating the penicillin with staphylococci at 37°C.

12. The adsorption of penicillin to stpahylococci was measured using the bio assay method.

13. The effects of Mg^{++} and Ca^{++} on penicillin adsorption to staphylococci were studied.

14. Dimethoxyphenyl penicillin was used in the pour plate method of testing for penicillin sensitivity in place of penicillin G. No inoculum effect was observed.

15. The lethal effects of combinations of Dimethoxyphenyl penicillin and penicillin G on staphylococci was studied in vitro.

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PENICILLIN RESISTANT STAPHYLOCOCCI

INOCULUM EFFECT

by

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An abstract of a thesis submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

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A number of strains of Staphylococcus aureus have been isolated by blood cultures from human infections and kept for this study. All of these strains have been bacteriophage typed with no common grouping noticeable.

The "inoculum effect," which deals with the increased resistance of staphylococci to penicillin by increased numbers of organisms in the inoculum, has been demonstrated by disk sensitivity testing methods. The inoculum effect can be of clinical importance when penicillinase producing staphylococci are tested for penicillin sensitivity by any of the commonly used methods (disk, pour plate, and tube dilution).

Pour plate methods of testing for the inoculum effect have been described. These tests clearly illustrate the inoculum effect. From these tests a direct relationship is seen between the number of organisms in the inoculum and the degree of penicillin resistance of a number of strains of staphylococci.

By various methods attempts were made to demonstrate that a culture may consist of both penicillin resistant and penicillin sensitive cocci. A culture of this mixture with the resistant cocci protecting the sensitive ones could explain the inoculum effect. Protection of this type could not be demonstrated.

Combination tube and pour plate methods of penicillin sensitivity testing demonstrated a stasis period in cell

reproduction, after which reproduction occurs at a normal rate. The length of this static period varies indirectly with the number of organisms in the inoculum.

A biological assay for penicillin was used to determine residual penicillin. It was found that the staphylococci destroy penicillin at quite a rapid rate. The penicillin concentration in the surrounding medium drops to a low level which will allow cell growth.

The penicillin assay method was also used to study the penicillin adsorption of the staphylococcal cells. It was found that 10^6 cells will bind approximately ten units of penicillin under the conditions outlined in the experiment. It is felt that a combination of penicillin destruction by the staphylococcal cells and the adsorption of the penicillin by the cells can explain the inoculum effect and the stasis period in the combination tube and pour plate test.

A new penicillin, dimethoxyphenyl penicillin which resists the action of penicillinase, was used in the pour plate method of testing for penicillin sensitivity and no inoculum effect was observed. An in vitro study was made to investigate the lethal effects of combinations of dimethoxyphenyl penicillin and penicillin G on staphylococci. This experiment suggests a potentiation of killing effect between the two penicillins.